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EFFECTS OF LIQUID STORAGE AND CRYOPRESERVATION ON PLATELET SURFACE GLYCOPROTEINS, LIGHT SCATTER, AND PROCOAGULANT ACTIVITY

BY

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ABSTRACT

This study was designed to examine the effects of standard 22°C platelet storage and platelet cryopreservation on surface glycoproteins, light scattering properties and surface-bound factor V using flow cytometry. Single donor platelet units were collected in ACD (formula A) using a Haemonetics Mobile Collection System. The units were either liquid-preserved at 22°C with rotation for up to 7 days or were cryopreserved using 6% DMSO as the cryoprotectant at -80°C. Storage at 22°C for 7 days resulted in an increased platelet-derived microparticle (PDMP) content which actively bound coagulation factor V in the absence of added agonist. Addition of 2 U/mL thrombin or a combination of 20 μM ADP and 20 μM epinephrine in the presence of 3 mM calcium resulted in an increased production of PDMP on days 5 and 7 compared to the day of collection. Plasma factor V activity of both the platelet unit and autologous ACD plasma decreased with 22°C storage, although the decrease was greater in the platelet product. Platelet surface P-selectin increased progressively during the 7 day 22°C storage period with a parallel decrease in responsiveness to the *in vitro* addition of 2 U/mL thrombin or 0.5 μM U46619 (a thromboxane A_2 analog). Platelet surface glyco-protein (GP) Ib decreased during 22°C storage, but GPIX, which is complexed with GPIb, did not change. Platelet surface GPIV increased slightly and the GPIa-IIa complex decreased slightly with 22°C storage. While constitutive expression of the platelet surface GPIIb-IIIa complex (measured by antibody 7E3) doubled over 7 days of 22°C storage, the exposure of the functional fibrinogen binding site on GPIIb-IIIa with the addition of agonist (measured by antibody PAC1) decreased dramatically during this same time period. This decreased reactivity to agonist was largely the result of an emerging sub-population of unresponsive platelets which could be readily observed by the bimodal distribution of the flow cytometry histograms of PAC1 specific fluorescence. Platelet cryopreservation resulted in the formation of PDMP post-thaw similar to the 7 day 22°C storage. A portion of these PDMP were lost after washing the thawed cryopreserved platelet unit, a step required to remove DMSO from the transfusion product. In contrast, the freeze-thaw process did not stimulate the formation of platelet microaggregates. Platelet surface P-selectin decreased slightly during the initial 22°C 24 hr testing period prior to freezing and increased significantly post-thaw. The platelet responsiveness to thrombin as measured by surface P-selectin decreased after the cryopreservation procedure. Cryopreservation also resulted in a decreased surface expression of GPIb, which was at least partially due to the development of a subpopulation of platelets with reduced surface GPIb. This subpopulation could be separately analyzed from the GPIb normal platelets, and, using 3 color flow cytometry, was shown to possess relatively higher amounts of surface bound factor V after the platelets were thawed. The GPIb reduced subpopulation was also less responsive to thrombin, as reported by surface P-selectin, than the GPIb normal platelets in the same sample. In conclusion, flow cytometry based assays coupling light-scattering properties and multiple immunologic probes directed at functional sites on the platelet surface is a promising technology for the determination of pre-transfusion plateletpheresis product quality.

INTRODUCTION

The availability of monoclonal antibodies directed against well characterized functional platelet receptors combined with the use of flow cytometry allows the rapid analysis of many aspects of platelet pathophysiology.[1] Testing plateletpheresis products with a panel of antibodies will provide a comprehensive "picture" allowing assessment of the overall quality. Addition of *in vitro* agonists allows the study of platelet responses that result in receptor modulation and binding of coagulation and adhesive proteins. Selective evaluation of a variety of platelet properties, many of which can be analyzed simultaneously given the multiple-parameter capabilities of flow cytometry, promises to lead to predictive assays of *in vivo* platelet survival and function.

In this report, we used flow cytometry to study two methods of plateletpheresis product storage: conventional room temperature liquid storage and cryopreservation with dimethylsulfoxide as the cryoprotectant. A number of major platelet surface glycoproteins were studied including receptors for fibrinogen, fibronectin, thrombospondin, collagen, and von Willebrand factor. Modulation of the activation-dependent alpha-granule protein P-selectin involved in platelet-leukocyte interactions were examined. Particular emphasis was placed on platelet membrane alterations which result in light scattering changes and binding of coagulation factor V. These properties have been used to describe the appearance of platelet-derived microparticles found in fresh single-donor platelets, liquid stored plateletpheresis units, and cryopreserved plateletpheresis units.

Previous *in vitro* studies by Sims and Shattil [2,3] have shown that platelet activation results in release of platelet-derived microparticles that bind activated coagulation factor V to a much greater extent than platelets. Activation-induced platelet-derived microparticles are therefore considered to play a central role in assembly of the coagulation system and to be "procoagulant". Bode et al.[4] have demonstrated that storage of plateletpheresis products in the blood bank results in a time-dependent increase in platelet-derived microparticles. However, it is not known whether these storage-induced platelet-derived microparticles also bind factor V.

METHODS

Plateletpheresis Procedure: Plateletpheresis products were collected at the Naval Blood Research Laboratory from normal donors meeting the requirements of AABB for healthy blood donors. None of the participants had taken medication for ten days prior to donation. Samples of peripheral blood were drawn from the donor in sodium citrate and acid citrate dextrose (ACD) anticoagulant (Vacutainers, Becton-Dickinson, San Jose CA) prior to plateletpheresis. Platelets were collected using ACD formula A anticoagulant and the Haemonetics Mobile Collection System (MCS, Haemonetics Corp., Braintree, MA). The products were sampled and put into 1000 ml CLX (tri-[2-ethyl-hexyl] trimellitate polyvinylchloride) platelet storage bags (Baxter, Deerfield, IL). For liquid preservation, the plateletpheresis product was placed on an Eberbach shaker at 140 oscillations per minute at 22°C for up to 7 days. Platelet products which were cryopreserved were kept at 22°C for the initial 24 hour period and frozen at -80°C in the presence of 6% dimethylsulfoxide (DMSO).[5]

Peripheral blood platelet-rich-plasma (PRP) or plateletpheresis product samples were collected and assayed by flow cytometry.[1,2,6,7] A total sample volume of <200 µl was required to perform the following determinations: a) platelet aggregate formation, b) the number of platelet-derived microparticles, c) the number of degranulated platelets (determined by platelet surface P-selectin), d) platelet surface GPIb, e) platelet surface GPIX, f) platelet surface GPIIb-IIIa, g) activated GPIIb-IIIa (reflecting exposure of the fibrinogen binding site), h) platelet surface GPIV, i) platelet surface GPIa-IIa and j) platelet

reactivity *in vitro* (determined by increased platelet surface expression of P-selectin, activated GPIIb-IIIa, surface bound coagulation factor V and decreased platelet surface expression of GPIb).

Plasma Coagulation Factor V Assay: Plasma factor V activity was measured using a Coag-A-Mate X2 automated coagulation system (General Diagnostics, Durham, NC). Citrate anticoagulated plasma samples were stored at -20°C and thawed in a 37°C water bath prior to the batch run. Samples were rapidly thawed to prevent denaturation of plasma fibrinogen. Test plasma samples were tested for their ability to correct the prothrombin time of factor V deficient plasma (# 38919, General Diagnostics) after addition of tissue thromboplastin plus ionized calcium (# 35120 Simplastin, General Diagnostics). Four dilutions of normal plasma provided a standard curve of clotting times from which the test plasma activity was derived. Units are % Factor V specific clotting activity, the normal range for human citrate plasma is 40-150%.

Platelet Cryopreservation: The cryopreservation method used for this study has been previously described in detail.[5] Briefly, the 24 hour plateletpheresis products were transferred into a 1000 mL PVC platelet freezing bag (Fenwal 4R2986, Deerfield, IL). A volume of 50 mL of 27% DMSO in saline was added at room temperature over a 5 minute period to achieve a final concentration of 6% DMSO. The platelets were placed in an aluminum container and kept at -80°C to achieve a freezing rate of 2-3°C per minute and stored at -80°C. The platelets were thawed in a 42°C water bath and

diluted with a 250 mL volume of 0.9% NaCl, 0.2% glucose, 40 mg% inorganic phosphorus, pH 5 (Cytosol Laboratories, Braintree, Ma). The platelets were then washed by centrifugation at 4500 x g for 5 minutes, removing the supernatant (eliminating 95% of the DMSO) and resuspension in previously-frozen autologous ACD plasma.

Flow cytometry: ACD anticoagulated peripheral blood samples obtained prior to plateletpheresis were centrifuged for 10 minutes at 150 x g and the PRP supernatant removed. The flow cytometry sample processing methods used in this study were essentially as previously described.[7,8] Peripheral blood PRP and unit samples were incubated (22°C, 15 minutes) with 2 U/mL thrombin (gift of Dr. John Fenton) in the presence of 2.5 mM gly-pro-arg-pro (GPRP, an inhibitor of fibrin polymerization purchased from Calbiochem, San Diego, CA) the stable thromboxane analogue U46619 (0.5 μM) or modified HEPES-Tyrodes (HT) buffer only (137 mM NaCl, 2.8 mM KCl , 1 mM $MgCl_2.6H_2O$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 5.5 mM Glucose, 10 mM HEPES and 0.35% bovine serum albumin, All buffer components were purchased from Sigma, St. Louis MO). Samples were then fixed by adding 1% formaldehyde (ultrapure methanol-free formaldehyde purchased from Polysciences, Warrington PA) for 30 minutes at 22°C, diluted 10-fold with HT buffer and stored at 4°C until labeled with monoclonal antibodies for flow cytometry. Aliquots of this preparation were incubated with anti-CD61-FITC (DAKO, Carpinteria CA) at a near saturating concentration and biotin conjugates (biotinylated in our lab using biotin-X-NHS, purchased from Calbiochem) of either one of the following

murine monoclonal antibodies at a saturating concentration for 15 minutes at 22°C, S12 (gift of Dr. R. McEver), 6D1 (gift of Dr. B. Coller), TM60 (gift of Dr. N. Yamamoto), AN51 (R-phycoerythrin direct conjugate, purchased from DAKO), WM23 (gift of Dr. M. Berndt), FMC25 (gift of Dr. M. Berndt), OKM5 (purchased from ORTHO Diagnostics, Rariton NJ), 6F1 (gift of Dr. B. Coller) or the equivalent concentration of isotypic control murine IgG (Sigma). This was followed by adding streptavidin-phycoerythrin (Jackson Immunoresearch Labs, West Grove PA) for an additional 15 minutes at 22°C. Samples were then diluted 10-fold and analyzed in a Coulter Profile flow cytometer. DNA check and standard bright flow cytometry beads purchased from Coulter were used for daily instrument calibration. Appropriate color compensation was set for fluorescence 1 and fluorescence 2 using 525 and 575 bandpass filters respectively. All data was saved in flow cytometry histogram files and analyzed using Coulter Elite software version 2.21. Platelets were identified based on binding of the anti-CD61-FITC antibody (fluorescence 1). Biotinylated test antibody binding expressed as linear mean fluorescence was collected based on phycoerythrin fluorescence (fluorescence 2). Percent of control fluorescence data was normalized to the daily peripheral blood sample after treatment with maximal thrombin (2 units/mL) for S12 and after treatment with buffer only for 6D1, TM60, WM23, AN51, FMC25, 6F1 and OKM5. In some cases the number of 6D1 (anti-GPIb) antibody molecules were determined by use of the "Quantum Simply Cellular Microbead Kit" (Sigma). Bead samples were processed exactly as the fixed platelets with the exclusion of the CD61-FITC antibody. Known numbers of anti-mouse-antibodies are present in a mixture of 4 bead preparations (plus a blank) allowing for

generation of a standard curve of fluorescence vs. number of antibodies. Fluorescence values from the platelet samples can thereby be expressed as number of antibody bound when read off the curve.

Three color flow cytometry samples utilized biotin-conjugated test antibodies (S12 directed against P-selectin or V237 directed against surface-bound coagulation factor V) at saturating concentration, R-phycoerythrin-conjugated anti-CD41/61 (7E3, a gift of Dr. B. Coller) at near saturating concentration, and FITC-conjugated 6D1 directed against GPIb at saturating concentration (followed by streptavidin-RED613 purchased from GIBCO-BRL, Grand Island NY). Activated and fixed samples prepared as described were labeled with 6D1, anti-CD41/61 and biotinylated S12 followed by streptavidin-RED613. For surface bound factor V analysis, anti-GPIb antibody, anti-CD41/61, GPRP (2 mM), autologous plasma (used as a source of coagulation factor V; plasma was prepared on the day of collection and stored with the platelet concentrate at 22°C in a satellite bag), unit or peripheral blood was incubated 10 minutes at 37°C with calcium ionophore A23187 (20 μM) plus 3 mM $CaCl_2$ or HT buffer only. This was followed by the addition of V237-biotin or irrelevant mouse IgG-biotin and a 10 minute room temperature incubation. This was followed by streptavidin-RED613 addition and a 10 minute room temperature incubation. Finally, samples were fixed by the addition of 1% ultrapure formaldehyde and a 30 minute room temperature incubation. Samples were then diluted 20 fold with HT buffer and kept at 4°C until run on the EPICS Profile flow cytometer within 24 hours. The factor V binding subpopulation method for platelet-derived microparticle determination was analyzed by a triangular region (see figure 2 below) which selected events which were both high for

bound factor V fluorescence and reduced in log forward light scatter signal. Two color samples prepared for factor V analysis were treated similarly with the omission of the biotinylated antibody (and streptavidin reagent) and use of FITC-conjugated V237. Activated GPIIb-IIIa (antibody PAC1, a gift from Dr. S. J. Shattil) samples were also processed similarly except FITC-conjugated PAC1 was added prior to activation, and CD41/61-R-phycoerythrin was added after activation to avoid any possible blocking of PAC1 binding. Samples were discriminated by the CD41/61-R-phycoerythrin signal and light scatter gates were set to include both platelets and platelet derived microparticles. For 3 color samples containing bimodal GPIb populations, the GPIb normal and low populations were analyzed separately. Fluorescence data was collected using 525, 575 and 625 bandpass filters for FITC, R-phycoerythrin and RED613 (a Texas red/phycoerythrin energy coupled dye) respectively. 3 color controls were prepared with maximal and minimal fluorescence signals in combination to set electronic color compensation.

Platelet aggregates were determined by flow cytometry, using the log forward light scatter (LFS) parameter as a measure of platelet size.[6] Only those particles that bound the CD61-specific antibody (platelet-specific) were analyzed. Percent aggregates in the sample were calculated by analyzing positive LFS events using the peripheral blood sample as the control.

Platelet-derived microparticles were also determined by flow cytometry, using log forward light scatter. Beads of 0.71 and 0.83 μm diameter (Duke Scientific, Palo Alto, CA) were used to establish an arbitrary cutoff point in the log forward light scatter parameter

(see figure 1 below). Platelet-derived microparticles were quantitated by making an analysis region between the 0.71 μm and 0.83 μm bead peaks and recording all events which fell below this point.

Light scatter plots shown in the figures below were made by either preparing metafiles with Coulter Elite® software modified in the Drawperfect® graphics program, or by Isocontour® histograms imported into Sigmaplot®. All graphs were made using Sigmaplot for Windows version 2.

RESULTS

Figure 1 illustrates one method of analysis of platelet-derived-microparticles (PDMP). Panel A is a log forward vs. log side light scatter histogram of a mixture of 0.71 and 0.83 μm diameter polystyrene green-fluorescing beads (Duke Scientific, Palo Alto, CA). An analysis region was established starting between the 0.71 and 0.84 μm bead peaks and including all events from this point down on the log forward light scatter (LFS) Y-axis scale. Panels B through D illustrate the progressive increase in this region of platelet-specific events (positive for an anti-CD41/61 monoclonal antibody) with storage at 22°C with rotation. There was an increase in the low LFS region from 13% of all platelet-specific events on Day 0 to 35% on Day 7 of storage.

Figure 2 illustrates the factor V binding method for measuring PDMP. There was a concomitant increase in surface bound coagulation factor V (X axis) and a decrease in LFS (Y axis) in a subpopulation of platelet-derived events with either time in storage (panel D) or addition of exogenous agonist (panels B, C, E, and F). Those events which fell in the analysis region in the lower right of each histogram are PDMP positive for surface factor V. These PDMP with factor V bound to their surface are procoagulant, because factor V binding reflects assembly of the coagulation system.[9]

Figure 3 shows a direct comparison of the 2 methods of PDMP measurement on parallel samples. The forward light scatter method, employing bead standards (figure 3, square symbols) measured a 21.7% increase in PDMP by day 7 of 22°C storage of plateletpheresis products. The factor V binding subpopulation method (figure 3, round

symbols) measured a 21.1% increase in PDMP by day 7. All points are the results obtained in the absence of added agonist or calcium. The close agreement between these methods illustrates that the PDMP formed during 22°C storage, (measured with or without anti-factor V antibody) represents a subpopulation actively binding coagulation factor V. The forward light scatter method starts at approximately 13% on day 0 due to the fact that the arbitrary cutoff produced by using a region based on 0.71 μm beads includes some normal platelets in addition to PDMP. Using the factor V binding method however, the percent PDMP is nearly zero in a fresh platelet preparation.

The factor V binding method was used in the averaged data depicted in figure 4. The baseline % PDMP in the absence of added agonist or calcium (open bars) can be seen increasing with time of 22°C storage as in figure 3. The effect of added agonist is shown by the hatched bars. The response to thrombin, 2 U/mL or ADP plus epinephrine, 20 μM each, in the presence of 3 mM $CaCl_2$ are nearly the same (figure 4 rising right and left crosshatched bars respectively for thrombin and ADP/epinephrine). The capacity of the plateletpheresis products to generate PDMP in response to these physiologic agonists increases dramatically with time of storage. This effect can also be observed in the representative histograms in figure 2, where the day 7 panels E and F clearly contain greater numbers of PDMP than the day 0 panels B and C. The response to calcium ionophore A23187 (20 μM) in the presence of calcium can be seen in figure 4 (double cross-hatched bars). Since greater than 90% of the fresh platelets convert to factor V binding PDMP in response to A23187 on day 0, this agonist is used

as the positive control. The conversion to PDMP in response to this non-physiologic agonist decreased somewhat on days 5 and 7 of 22°C storage.

The binding of the V-specific antibody to the platelet surface is dependent on an adequate supply of soluble factor V which is present in both plasma and platelet alpha-granules. The plasma added to satisfy this assay requirement was collected at the time of plateletpheresis and stored at 22°C with the platelet concentrate in a satellite bag according to standard blood bank procedures. The decrease in the PDMP response to A23187 on days 5 and 7 post collection (figure 4) could therefore be due to a change in the platelet membrane binding capacity for factor V or a relative lack of available soluble factor V. To address this question, we assayed the plasma from the satellite bag and plasma prepared from the plateletpheresis product for factor V activity on days 0, 5, and 7. Figure 5 clearly shows that while the factor V activity is normal on day 0 in both the plasma and platelet concentrate preparations, there is a significant decrease in this activity on days 5 and 7 post-collection. Furthermore, the decrease is greater in the plateletpheresis product than in the plasma preparation (compare figure 5 crosshatched bars to open bars). The amount of factor V activity in the day 7 plateletpheresis product could, in fact be much less than shown because the lower limit of detection of the assay is 12.5%. The decrease in total PDMP generated by A23187 in figure 4 (double crosshatched bars) is therefore most likely due to a limiting concentration of soluble factor V rather than a change in the platelet membrane capacity to bind the factor V.

Platelet surface expression of P-selectin (an integral alpha granule membrane protein[10]) was used to monitor platelet degranulation by flow cytometry. Figure 6 shows the progressive increase in surface P-selectin over time of 22°C storage in the absence of added agonist (round symbols). By day 7 nearly a third of the granule membrane content of P-selectin was present on the platelet surface. The light scatter gates were made large enough to include both intact platelets and PDMP. The response to thrombin (2 U/mL) stimulation decreased over time (triangular symbols), as did the response to the synthetic thromboxane A_2 analog U46619 (square symbols). The loss of U46619 response occurred more rapidly as can be seen by the day 5 data. The storage-dependent loss of response to thrombin and U46619 is in fact more profound, given the higher starting level of surface P-selectin on days 5 and 7. For example, the percent increase on day 7 is nearly zero for U46619 and only about 20% for thrombin.

The platelet surface glycoprotein (GP) Ib-IX involved in vWF mediated adhesion to damaged subendothielium[11] was measured by flow cytometry using a panel of 5 monoclonal antibodies. Four antibodies were directed at distinct epitopes on the alpha chain of GPIb (CD42b). As can be seen in figure 7, by day 7 of 22°C storage, binding of all 4 antibodies decreased in parallel to about 35% of the day 0 maximum. In contrast to GPIb, platelet surface GPIX (CD42a) as detected by antibody FMC25 is quite stable during 22°C liquid storage (figure 7).

Figure 8 shows the effect of 22°C storage on platelet surface GPIV and the GPIa-IIa complex as measured by flow cytometry. Both of these receptors have been implicated in platelet adhesion to collagen.[12,13] The surface GPIV increases about 30%

by day 7 of 22°C storage, while GPIa-IIa remains stable until day 5 and then decreases on day 7.

The GPIIb-IIIa complex (CD41/61, integrin $\alpha_{IIb}\beta_3$) is the highest density receptor on the platelet surface and binds fibrinogen when in the activated state.[14,15] Figure 9 depicts the binding of 2 antibodies directed against constitutive epitopes which represent the number of surface receptors regardless of activation state. These antibodies indicate a substantial increase in both the GPIIb-IIIa complex and the GPIIIa subunit with 22°C liquid storage. By day 7, the number of GPIIb-IIIa molecules has doubled compared to the day of platelet collection. The high surface density and increased surface expression with storage make GPIIb-IIIa the ideal surface protein for platelet identification under a variety of circumstances.

Activated GPIIb-IIIa undergoes a conformational change which allows the binding of fibrinogen, which in turn results in interplatelet cross-linking and aggregation.[14,15] Figure 10 reports the binding of antibody PAC1 which is directed against this fibrinogen binding site. In the absence of added agonist, binding of this antibody barely increased with time in 22°C storage (figure 10, round symbols). In contrast, 2 units/mL thrombin (figure 10, square symbols) or 20 μM ADP plus 20 μM epinephrine (figure 10, triangular symbols), each in the presence of 3 mM calcium caused a maximal exposure of the fibrinogen binding site on day 0. This response is approximately halved on day 5 of liquid storage and nearly absent by day 7, despite the elevated number of GPIIb-IIIa surface molecules (see figure 9). Since flow cytometry analyzes antibody binding on each individual platelet[1], decreased binding can be

characterized as a generalized shift in the entire population or development of an unresponsive subpopulation. The representative histograms in figure 11 show that both of these mechanisms are in operation to reduce the overall PAC-1 binding. In both donors shown, the day 0 peak is the brightest fluorescing (furthest to the right) compared to the other positive peaks. The day 5 and 7 histograms reveal an emerging subpopulation unable to respond to the added agonist, in this case ADP/epinephrine in the presence of 3 mM $CaCl_2$. Additionally, the positive portion of the day 5 and 7 histograms is lower than the day 0 peak indicating those still able to respond bind less antibody. Finally, comparison of the day 7 histograms from the 2 donors illustrates the donor variability in the ratios of responders to nonresponders. The majority of donor 2 day 7 platelets and PDMP are unresponsive to the agonist, whereas the opposite is true for donor 1 which is still largely responsive to ADP/epinephrine in the presence of 3 mM $CaCl_2$.

In addition to standard liquid storage of plateletpheresis products at 22°C, cryopreservation of platelets in 6% DMSO has also been studied. Figure 12 compares light scatter plots of these 2 types of platelet products. Log side light scatter on the X axis measures cell shape and granularity, while log forward light scatter measures cell size and refractive index. As seen in the upper 3 panels, the changes with liquid preservation are mainly in forward light scatter, with less side scatter shift until substantial PDMP formation has occurred. The cryopreserved platelets underwent little change pre-freeze (lower left panel), since they were frozen 24 hours after collection (the time required to complete the testing for the current FDA-mandated infectious

disease markers). However, dramatic changes can be observed in the post-thaw product (lower right panel). The post-thaw platelet concentrate decreases in the forward and side light scatter axes, with a larger increase in PDMP than is typically seen with liquid storage. Figure 13 shows platelet light scatter plots at each stage of the cryopreservation procedure. No changes are observed over the first 24 hours of liquid storage, even after the addition of 6% DMSO. Once the product is thawed, the wash step required to remove the DMSO also removes a portion of the PDMP. This can be seen by comparing the lower regions (M) of the lower 2 panels of figure 13.

Figure 14 also includes data illustrating the removal of a portion of the PDMP produced by cryopreservation (cross-hatched bars at the post-thaw and post thaw-wash points). Platelet derived microparticles (PDMP) were quantitated by the factor V binding method. Approximately half of the PDMP produced are lost during the wash step after thawing the plateletpheresis product. No significant PDMP were formed during any phase of the pre-freeze procedure. Aggregates were measured by comparing unit samples to the donors peripheral blood platelets to establish log forward light scatter (LFS) regions corresponding to platelet-specific events of increased LFS.[6] No aggregates were produced by either the plateletpheresis procedure or at any stage of the cryopreservation procedure (figure 14, open bars).

Significant aggregates were not routinely observed in either the 22°C liquid storage or the cryopreserved plateletpheresis products included in this report. However, for comparative purposes, we have included a light scatter plot of a platelet concentrate in which microaggregates did form during the plateletpheresis procedure.

Figure 15 illustrates the different light scatter patterns produced by flow cytometry when analyzing a normal plateletpheresis product (panel A) or a plateletpheresis product containing microaggregates (panel B). Peripheral blood drawn from the donor is used as a control to verify the light scatter profile of normal platelets. Note the characteristic "tail" found on the upper right of panel B which includes the platelet microaggregates, the largest of which are approximately the size of a white cell. These are not macroscopic aggregates, which are not detectable by flow cytometry. Frequently, microaggregates will disaggregate during the course of the first 24 hours of 22°C liquid storage with rotation (data not shown).

Cryopreservation does not appear to cause platelet aggregation (see figure 14), despite a degree of platelet activation evidenced by appearance of the α-granule membrane protein P-selectin. Presented in figure 16, platelet and PDMP surface P-selectin increased after thawing the cryopreserved platelet concentrate. The percent of platelets and PDMP positive for P-selectin increased slightly after washing the thawed product, suggesting that the PDMP removed by the wash step may have less surface P-selectin than the intact platelets. There was a slight, but reproducible decrease in surface P-selectin over the initial 24 hours at 22°C prior to freezing. After addition of 6% DMSO, but prior to freezing, a small increase in surface P-selectin was observed (figure 16). Figure 17 illustrates that cryopreservation caused a decrease in overall reactivity of platelets and PDMP to thrombin as measured by their upregulation of P-selectin following the *in vitro* addition of agonist (compare the cross-hatched bars).

The effect of cryopreservation on platelet and PDMP surface GPIb can be seen in figure 18. While GPIb remained normal through the 24 hour 22°C liquid storage testing period, the freeze-thaw process caused the overall surface GPIb to decrease to less than half the initial content. Figure 19 again illustrates the cryopreservation-induced decrease in responsiveness to thrombin, in this case as determined by the reduced degree of the thrombin-dependent decrease in platelet surface GPIb.[7]

The decrease in GPIb caused by the freeze-thaw process was further characterized by 3 color flow cytometry. The platelets and PDMP identified by binding of anti-CD41/61 antibody were further labeled with 2 test antibodies simultaneously. The 2 dimensional histogram shown in figure 20 plots bound factor V fluorescence vs. surface GPIb fluorescence. Separate populations can be distinguished that have normal surface GPIb (figure 20, region B) and reduced GPIb (figure 20, region A). Therefore, the overall reduction in platelet and PDMP surface GPIb reported in figure 18 can be accounted for by the emergence of a subpopulation which has markedly reduced GPIb. Furthermore, the reduced GPIb subpopulation in the thawed platelet concentrate has relatively more surface bound coagulation factor V (figure 20, region A). This subpopulation accounts for the majority of factor V binding PDMP such as those reported in figure 14.

The GPIb normal and reduced subpopulations produced by cryopreservation are distinctly separable, allowing comparative analysis. Figure 21 reports the number of PDMP represented by each of these 2 subpopulations. Prior to addition of agonist, approximately 90% of the GPIb reduced subpopulation have surface bound factor V

(figure 21, square symbols) and are reported as PDMP, while only a third of the GPIb normal subpopulation are factor V positive (figure 21, round symbols). Addition of calcium ionophore A23187 in the presence of exogenous calcium converts the majority of GPIb normal platelets to PDMP, while the GPIb reduced subpopulation which are already PDMP increase slightly with A23187 addition (figure 21).

The P-selectin or granule release response can also be compared using 3 color flow cytometry. The GPIb normal subpopulation retains the thrombin-induced degranulation response much better than the GPIb reduced subpopulation (compare the figure 22 round symbols to square symbols). Prior to addition of agonist, the measured surface P-selectin was slightly higher on the GPIb normal population compared to the GPIb reduced, although the difference was not significant.

To summarize many of the advantages of the multiparameter analysis capabilities of flow cytometry, fresh and post-thaw cryopreserved platelet products are compared in figure 23. Each of these products were labeled with 3 antibodies coupled with fluorophores emitting light at different wavelengths after excitation with a single standard argon laser. Each of the 2 sets of 4 histograms were produced from a single sample. While differences are not immediately apparent by side light scatter or constitutive GPIIb-IIIa fluorescence, both GPIb and factor V fluorescence reveal the obvious differences which have been detailed in the preceding paragraphs.

DISCUSSION

In this study, we used factor V binding to platelet-derived microparticles (PDMP) as a marker of the assembly of the coagulation system on microparticles, to address the following hypotheses: 1) Does storage of plateletpheresis products generate PDMP that bind factor V? 2) Do stored platelets have the capacity to generate additional PDMP that bind factor V? To accomplish this, we used flow cytometric methods. To minimize artefactual *in vitro* formation of PDMP as a result of experimental manipulation, we studied PDMP without separation of platelets and/or PDMP from the plasma milieu of plateletpheresis products. In addition to the factor V binding method, we employed a method using log forward light scatter (LFS) alone, with microbead standards of known diameter (similar to previous reports[4]).

Studies reported here confirm that the liquid storage of plateletpheresis products resulted in production of microparticles measurable by either of the above methods. Factor V binding studies showed these to be more procoagulant than the intact platelets in the same sample. Addition of either thrombin plus calcium or ADP plus epinephrine and calcium demonstrated that these stored plateletpheresis products had an increased capacity to generate additional procoagulant PDMP to these physiologic agonists. Total binding of factor V was achieved by addition of calcium ionophore A23187 in the presence of exogenous calcium. The decreased levels of binding on days 5 and 7 was most likely due to the lack of soluble factor V available in the day 5/7 plasma stored at 22°C.

P-selectin is an alpha-granule membrane protein which becomes surface expressed platelet activation and secretion.[10] This receptor is responsible for the adhesion of platelets-to monocytes and neutrophils.[16,17] The surface P-selectin decrease over the first 24 hours of 22°C storage observed in this study (figure 15) is difficult to explain, since the exposed membrane of the platelet α-granules is not thought to be re-internalized after degranulation. One possible explanation, is the binding of a subpopulation of P-selectin positive platelets to the small number of contaminating leukocytes present in the platelet unit. This would result in their not being analyzed 24 hours after collection as single platelets, and would lower the percent of individual platelets positive for P-selectin. In contrast to the 24 hour change, the platelet surface expression of P-selectin increased with longer 22°C storage. This would most likely lead to leukocyte adhesion *in vivo* upon transfusion of the platelet unit. There was also a decreased platelet reactivity with 22°C storage, as determined by surface exposure of P-selectin after treatment with U46619 or thrombin *in vitro*.

Glycoprotein Ib (GPIb) is required for initial platelet adhesion to damaged subendothielium.[18,19] Loss of this receptor results in the loss of this aspect of platelet function.[18,19] Storage at 22°C resulted in a progressive decrease in the platelet surface binding of a panel of anti-GPIb antibodies. The fact that all 4 antibodies decreased in parallel strongly suggested this decrease was not the result of a conformational change in the molecule, but cleavage of the majority of the extracellular portion of the receptor. Increased plasma glycocalicin has been correlated with decreased platelet surface

GPIb during 22°C storage of plateletpheresis products.[20] GPIX, which is complexed to GPIb[18,19], did not decrease with liquid storage.

Platelet surface GPIV (CD36 or sometimes referred to as GPIIIb) has been found to be a receptor for both collagen [12,21] and thrombospondin.[22-24] The platelet surface complex GPIa-IIa (CD49b/29) has also been shown to have a role in collagen adhesion.[13] Platelet surface GPIV increased approximately 30% by day 7 of 22°C storage, whereas GPIa-IIa was slightly reduced by day 7.

The platelet GPIIb-IIIa complex (CD41/61, $\alpha_{IIb}\beta_3$ integrin) binds fibronectin, von Willebrand factor and most importantly fibrinogen, which is critical for platelet aggregation when in the activated state.[14,15] There are many constitutive epitopes which represent the number of surface receptors in the activated or unactivated state. By day 7 of 22°C storage the number of GPIIb-IIIa surface receptors doubled compared to the day of platelet collection. This was most likely the result of translocation from internal stores such as granule membranes and the surface connected canalicular system.[25,26] The functional state of GPIIb-IIIa can be measured by the antibody PAC1 which specifically occupies the fibrinogen binding site on activated GPIIb-IIIa.[15] While 22°C storage did not itself elevate this functional site, *in vitro* agonist challenge showed a progressive decrease in the platelet response resulting in exposure of the fibrinogen binding site. Further examination of the PAC1 histograms revealed an emerging subpopulation of unresponsive platelets (or PDMP) accounting for the majority of the decreased responsiveness.

Light scattering properties of cells analyzed by flow cytometry can provide valuable objective information regarding the structural state of stored plateletpheresis products. Light scattering has been employed to assess the general morphology of plateletpheresis products by automated methods of flowing material[27,28] analogous to visual examination of swirl. In contrast, flow cytometry provides averaged "snapshots " of the forward and side light scattering of thousands of *individual* platelets. While forward light scatter generally reflects cell size, we and others [29] have observed forward light scatter shifts in cells which have little or no change in cell volume. This may reflect an increase in membrane permeability which results in decreased forward light scatter due to a reduction in the refractive index between the cell and the surrounding medium. Light scattering shifts of this type reflect loss of membrane integrity which accompanies decreased viability. Therefore, while forward light scatter reduction during platelet storage may or may not reflect active vesiculation resulting in true microparticle formation, forward light scatter reduction due to increased membrane permeability provides additional information valuable for assessment of the quality of plateletpheresis products. The loss of some low forward light scatter events after washing a thawed cryopreserved plateletpheresis product is probably due to removal of PDMP of low density, leaving the larger platelets and PDMP. Small, macroscopically invisible aggregates, present in a plateletpheresis product can also be measured by flow cytometry by observing an increase in the forward light scatter signal. Although there were no aggregates of this type found in the plateletpheresis products in either the 22°C storage or the cryopreservation sections of this study, aggregates formed

during the plateletpheresis procedure are not uncommon. These can be formed due to platelet exposure to shear forces or too low a citrate level during collection, donor variability (hyperaggregabilty), or a combination of these factors. Plateletpheresis products containing these microaggregates are likely to also have elevated activated surface GPIIb-IIIa and P-selectin although these are difficult to measure accurately by flow cytometry since such measurements ideally require a single cell suspension.

The thawed, cryopreserved plateletpheresis products included in this study exhibited increased surface P-selectin compared to the pre-freeze level. While P-selectin mediates the adhesion of platelets to monocytes and neutrophils,[16,17] this does not necessarily cause them to be removed from the general circulation following transfusion.[30] The post-thaw cryopreserved plateletpheresis product is also less responsive to thrombin activation, perhaps partially as a result of loss of some GPIb receptors which have been implicated in localization of thrombin on the platelet surface[31,32] although GPIb is not the functional thrombin receptor.[33]

The freeze/thaw process also decreased the overall platelet surface GPIb, primarily due to the appearance of a subpopulation with greatly reduced surface GPIb. The specific epitope of the GPIb-specific monoclonal antibody 6D1 used in most of these studies is directed at the functional von Willebrand receptor.[34,35] The GPIb reduced subpopulation is clearly separable from the GPIb normal subpopulation allowing comparative analysis of the 2 populations by 3 color flow cytometry. The GPIb reduced population has increased surface bound coagulation factor V. Despite the reduced GPIb, transfusing these products may prove beneficial for the control of

bleeding because bound factor V reflects a procoagulant property.[2,36] The GPIb reduced population was however less responsive to thrombin as reported by the P-selectin-specific antibody S12.

The use of flow cytometric based multiparameter assays would allow more information about the condition of plateletpheresis products to be gathered with a minimum of time required by laboratory staff and little additional cost. Routine screening would improve the detection of suboptimal platelet products prior to their administration, thereby reducing the incidence of alloimmunization and transfusion related infections by reducing the number of required transfusions. Further *in vivo* studies will be required to determine which of these measurements are most likely to predict *in vivo* platelet survival and function.[30]

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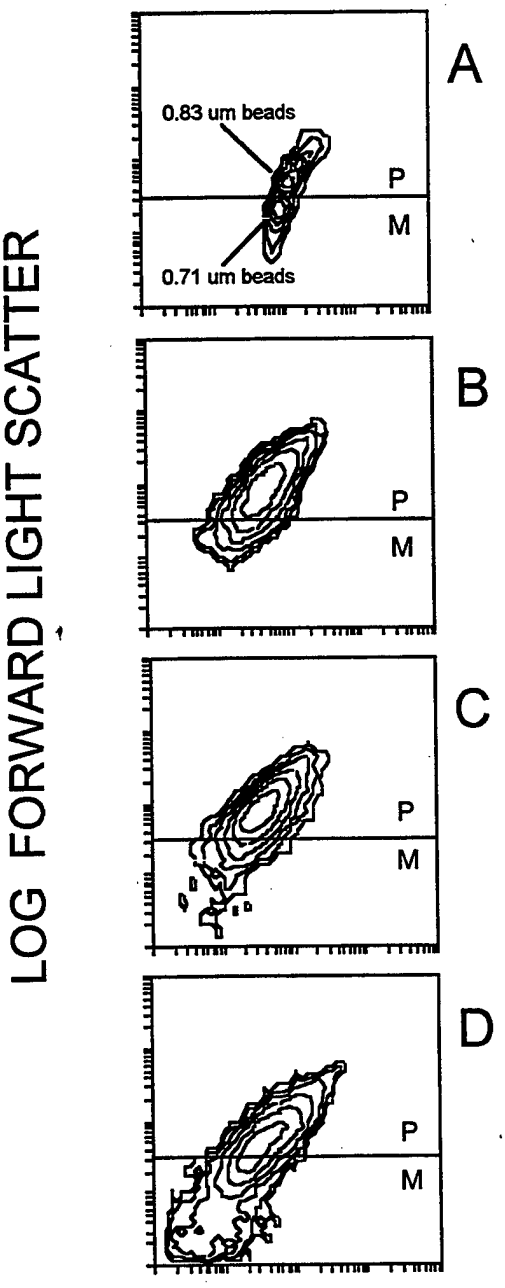
FIGURE 1

Representative light scatter histograms from 22°C liquid preserved plateletpheresis products. The top panel (A) shows bead standards of 0.83 and 0.71 µm diameter used to establish the log forward light scatter regions separating platelets (P, upper region of each histogram) and microparticles (M, lower region of each histogram). These regions remained for the subsequent platelet analysis on days 0, 5 and 7 (panels B, C, and D). Platelets and platelet-derived microparticles were identified with anti-CD41/61 antibody.

FIGURE 1



BEAD STANDARDS

DAY 0 UNIT

DAY 5 UNIT

DAY 7 UNIT

FIGURE 2

Representative light scatter vs. bound factor V fluorescence histograms from 22°C liquid preserved plateletpheresis products. Platelets and platelet-derived microparticles (PDMP) were identified with anti-CD61 antibody. Regions shown (triangular box in the lower right of each histogram) illustrate the log forward light scatter vs. factor V binding method of quantitating PDMP. The presence of factor V-bound PDMP, as defined by the binding of the factor V-specific monoclonal antibody, is apparent on day 7 of liquid storage in the absence of added exogenous agonist. Note the increased generation of factor V-positive PDMP on days 5 and 7 in response to 2 units/mL thrombin or a combination of 20 μM ADP and 20 μM epinephrine in the presence of 3 mM $CaCl_2$.

FIGURE 2

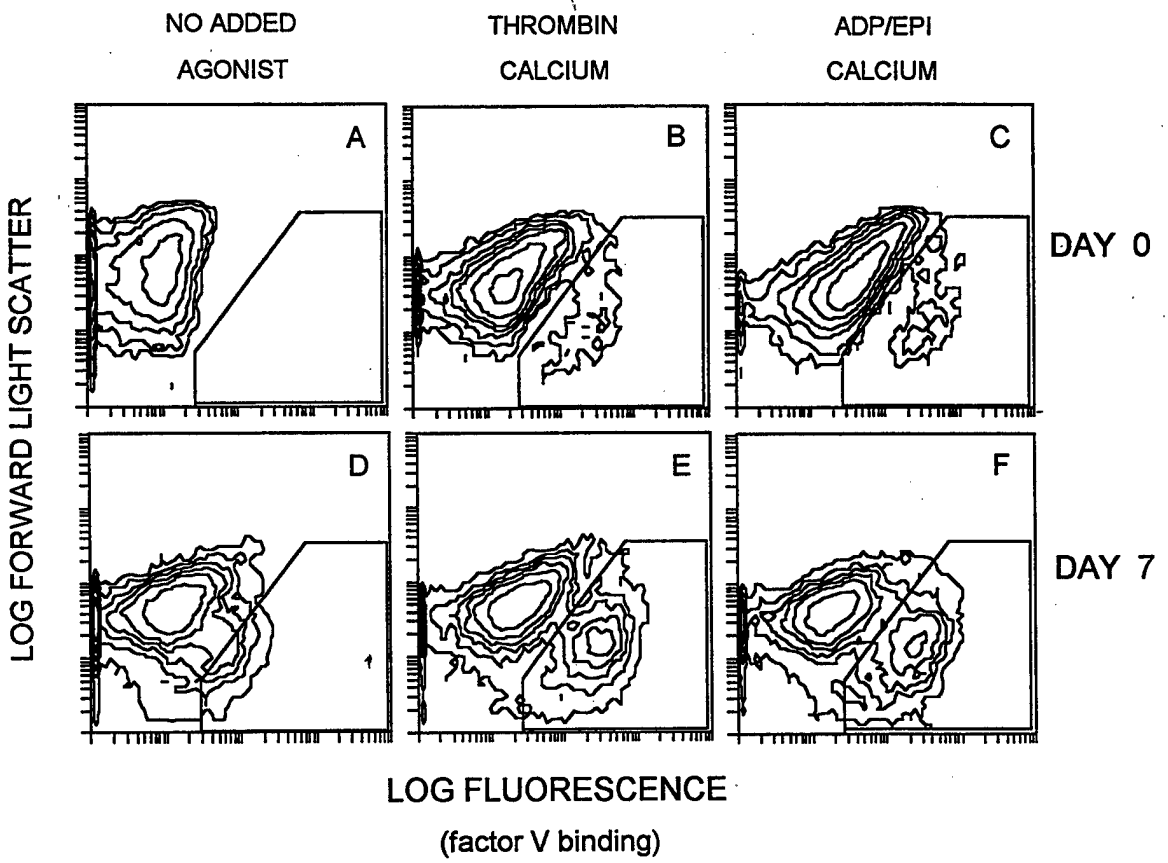

NO ADDED
AGONIST
THROMBIN
CALCIUM
ADP/EPI
CALCIUM
A
B
C
D
E
F
DAY 0
DAY 7
LOG FORWARD LIGHT SCATTER
LOG FLUORESCENCE
(factor V binding)

FIGURE 3

Formation of PDMP during 22°C liquid storage of plateletpheresis products. Platelets and PDMP were identified by binding of anti-CD41/61-R-phycoerythrin or anti-CD61-FITC conjugated monoclonal antibody. PDMP were identified by 2 methods: 1) binding of surface-bound factor V (see Fig. 2) and 2) decrease in log forward light scatter below a point defined by 0.71 and 0.83 μm diameter bead standards (see Fig. 1). Note the equivalent increases in percent microparticles measured by each method. Data are mean ± SEM, n=6.

FIGURE 3

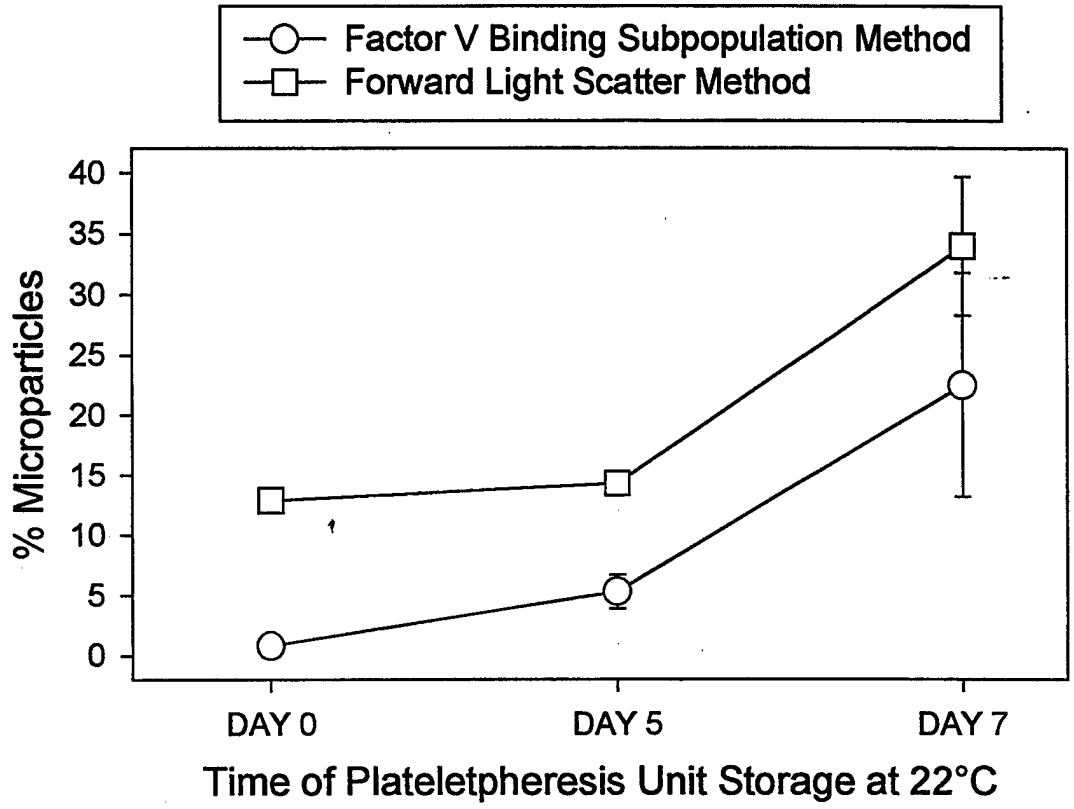

Factor V Binding Subpopulation Method
Forward Light Scatter Method
% Microparticles
40
35
30
25
20
15
10
5
0
DAY 0
DAY 5
DAY 7
Time of Plateletpheresis Unit Storage at 22°C

FIGURE 4

Percent PDMP, as determined by surface binding of factor V and decreased log forward light scatter. Platelets and PDMP were identified by binding of anti-CD61 monoclonal antibody. Data are mean ± SEM, n=6.

FIGURE 4

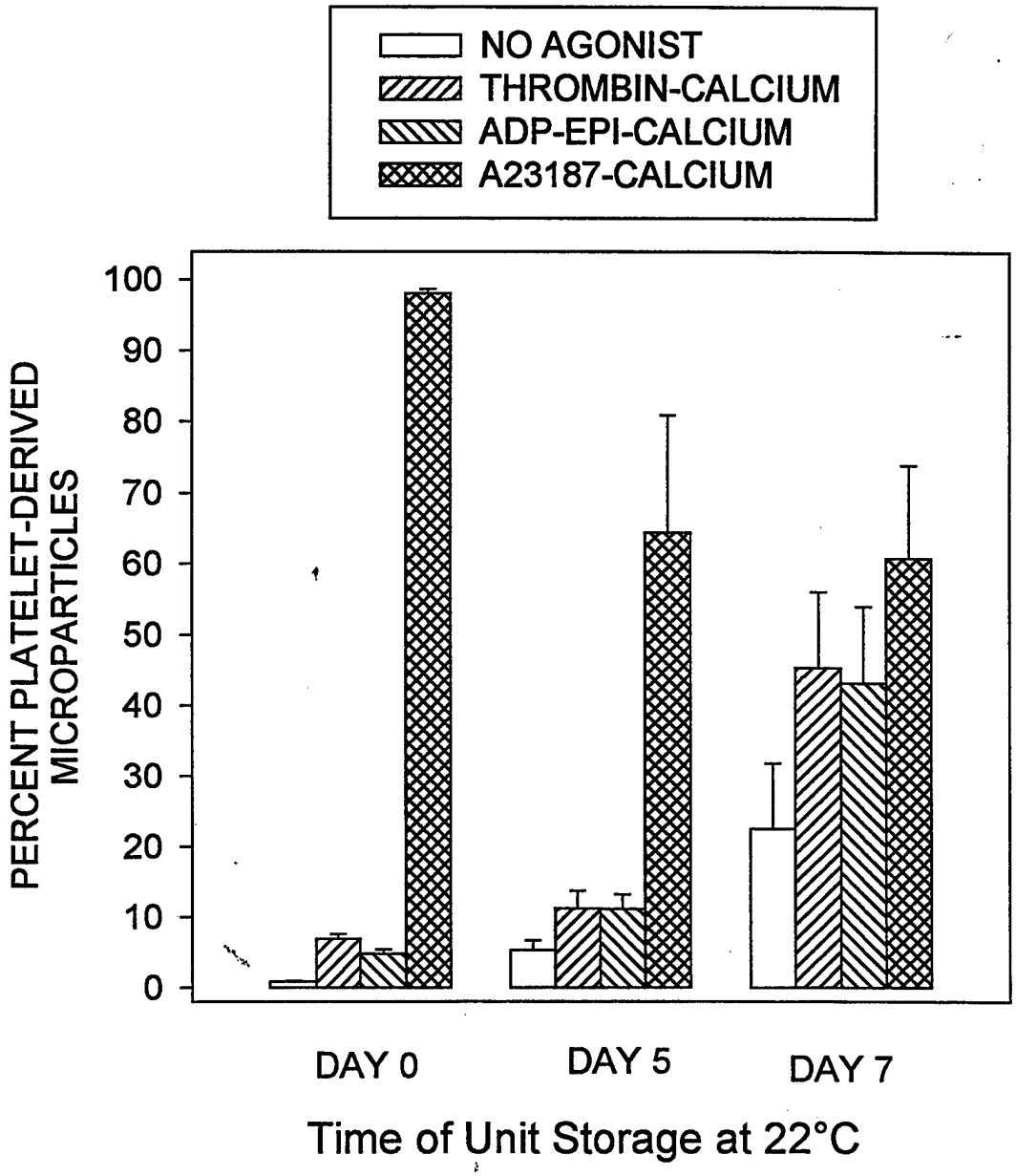

NO AGONIST
THROMBIN-CALCIUM
ADP-EPI-CALCIUM
A23187-CALCIUM
PERCENT PLATELET-DERIVED MICROPARTICLES
100
90
80
70
60
50
40
30
20
10
0
DAY 0
DAY 5
DAY 7
Time of Unit Storage at 22°C

FIGURE 5

Plateletpheresis units and autologous plasma were stored at 22°C with rotation. Aliquots were removed on days 0, 5 and 7 and the plasma assayed for soluble coagulation factor V activity. All day 7 unit samples were <12.5 (the lower limit of the standard curve) and could therefore be considerably lower than depicted. Data are mean ± SEM, n=4.

FIGURE 5

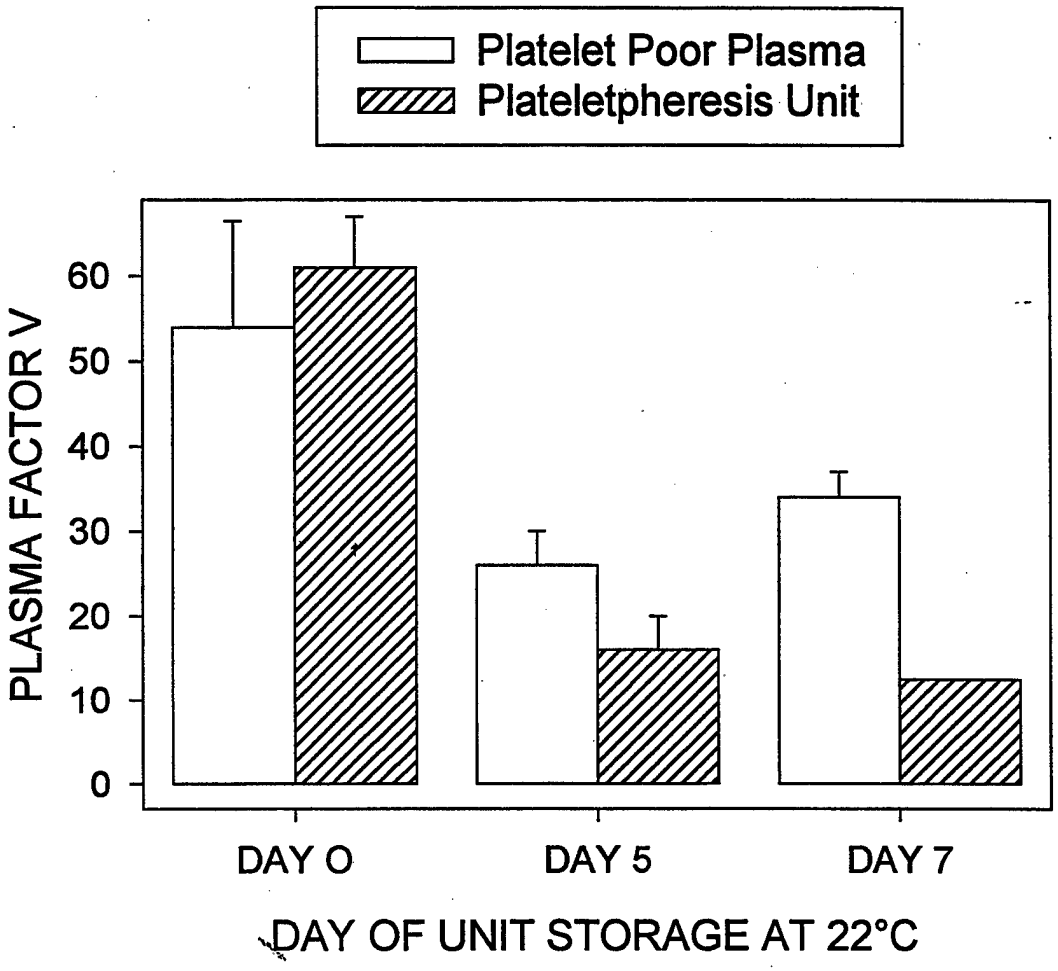

Platelet Poor Plasma
Plateletpheresis Unit
PLASMA FACTOR V
60
50
40
30
20
10
0
DAY O
DAY 5
DAY 7
DAY OF UNIT STORAGE AT 22°C

FIGURE 6

Effect of 22°C liquid storage on platelet surface P-selectin. Unit samples were incubated with the thromboxane A_2 analogue U46619, human α-thrombin (in the presence of GPRP) or no added agonist (buffer only) for 15 minutes. Platelets were identified with anti-CD41/61 antibody. Light scatter gates were set to include both platelets and PDMP. Maximally activated (2 U/mL thrombin) peripheral blood platelet S12 binding was assigned 100 fluorescence units. Data are mean ± SEM, n=6.

FIGURE 6

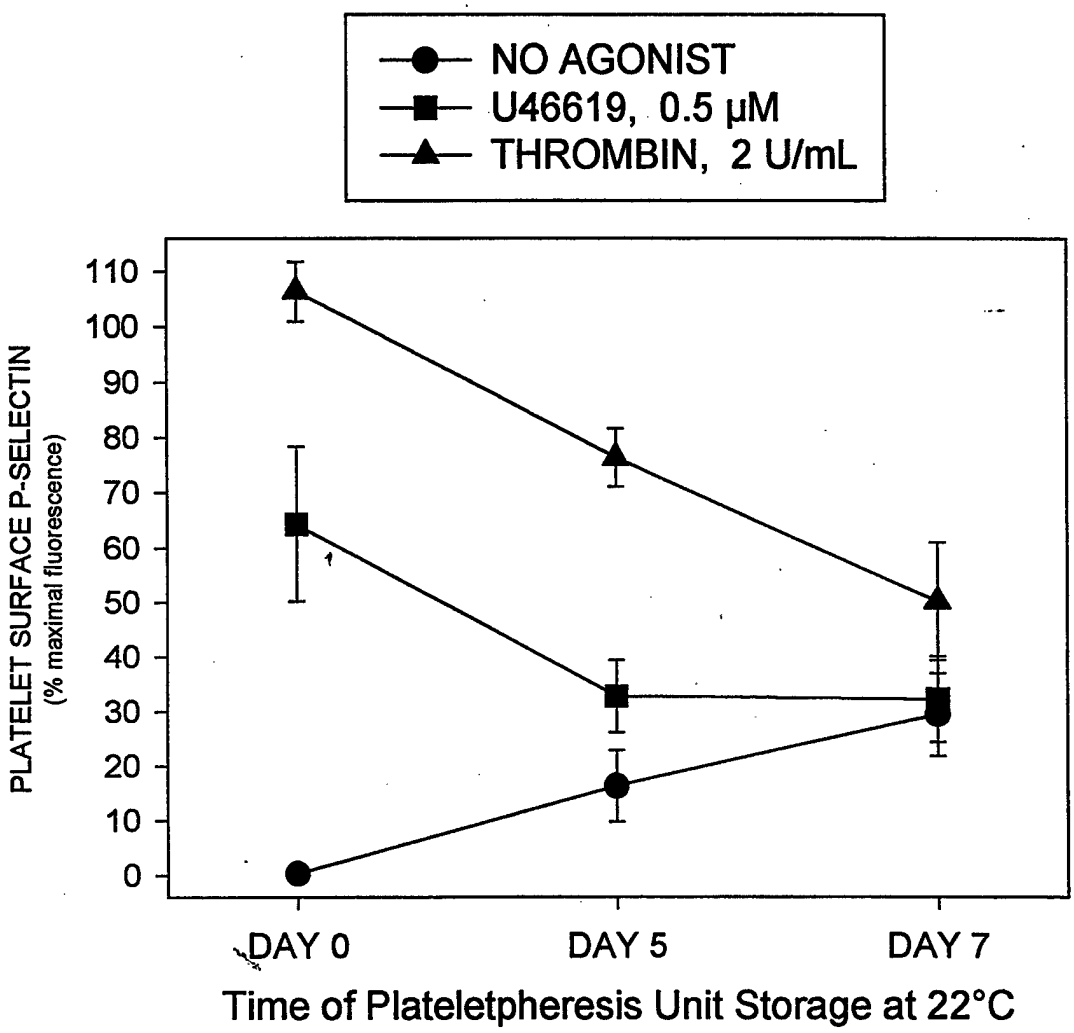

NO AGONIST
U46619, 0.5 µM
THROMBIN, 2 U/mL
PLATELET SURFACE P-SELECTIN
(% maximal fluorescence)
110
100
90
80
70
60
50
40
30
20
10
0
DAY 0
DAY 5
DAY 7
Time of Plateletpheresis Unit Storage at 22°C

FIGURE 7

Effect of 22°C liquid storage on platelet surface GPIb and GPIX. Platelet surface GPIb decreased as reported by a panel of 4 monoclonal antibodies directed against different sites on the receptor including the von Willebrand factor binding site (6D1). GPIX, which is complexed with GPIb, is not lost during platelet storage. Light scatter gates were set to include both platelets and PDMP. The day 0 unactivated unit sample was assigned 100 fluorescence units. Data are mean ± SEM, n=6.

FIGURE 7

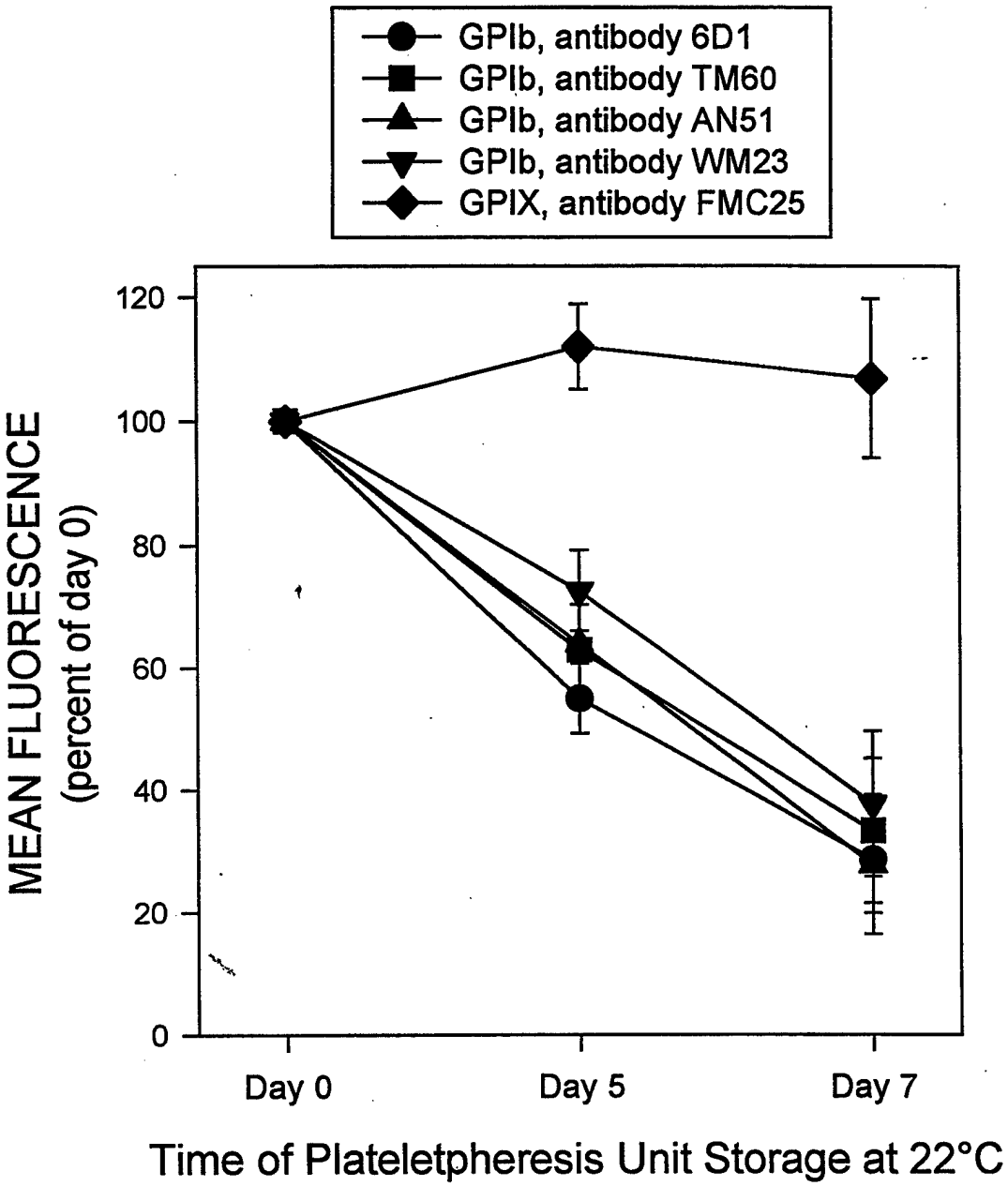

GPIb, antibody 6D1
GPIb, antibody TM60
GPIb, antibody AN51
GPIb, antibody WM23
GPIX, antibody FMC25
MEAN FLUORESCENCE
(percent of day 0)
120
100
80
60
40
20
0
Day 0
Day 5
Day 7
Time of Plateletpheresis Unit Storage at 22°C

FIGURE 8

Effect of 22°C liquid storage on platelet surface collagen receptors; GPIV and the GPIa-IIa complex. Platelets were identified with anti-CD41/61 antibody. Light scatter gates were set to include both platelets and PDMP. The day 0 unactivated sample was assigned 100 fluorescence units. Data are mean ± SEM, n=6.

FIGURE 8

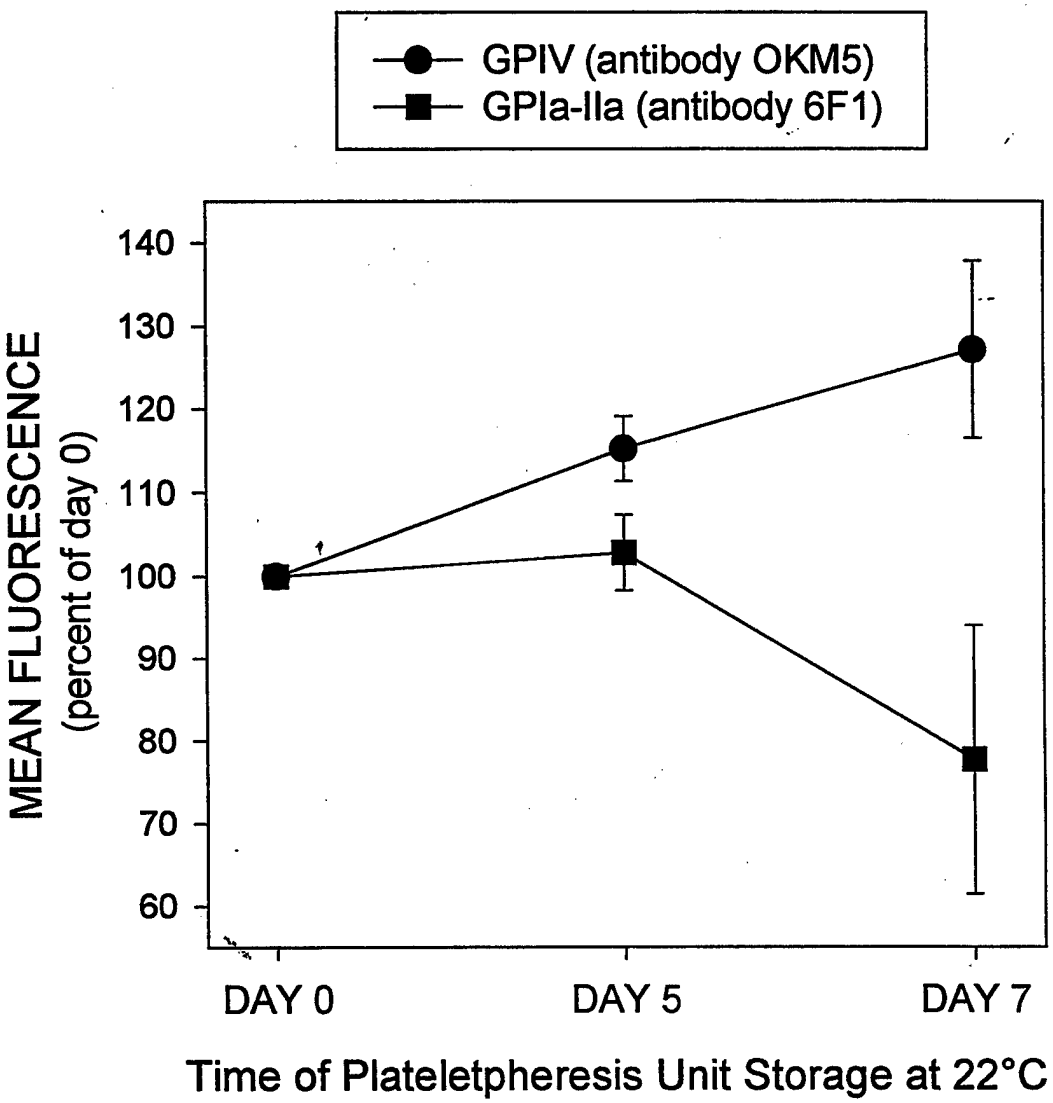

GPIV (antibody OKM5)
GPIa-IIa (antibody 6F1)
MEAN FLUORESCENCE
(percent of day 0)
140
130
120
110
100
90
80
70
60
DAY 0
DAY 5
DAY 7
Time of Plateletpheresis Unit Storage at 22°C

FIGURE 9

Effect of liquid storage on the platelet surface expression of the GPIIb-IIIa complex and the GPIIIa subunit. Light scatter gates were set to include both platelets and PDMP. The day 0 unactivated unit sample was assigned 100 fluorescence units. Data are mean ± SEM, n=6.

FIGURE 9

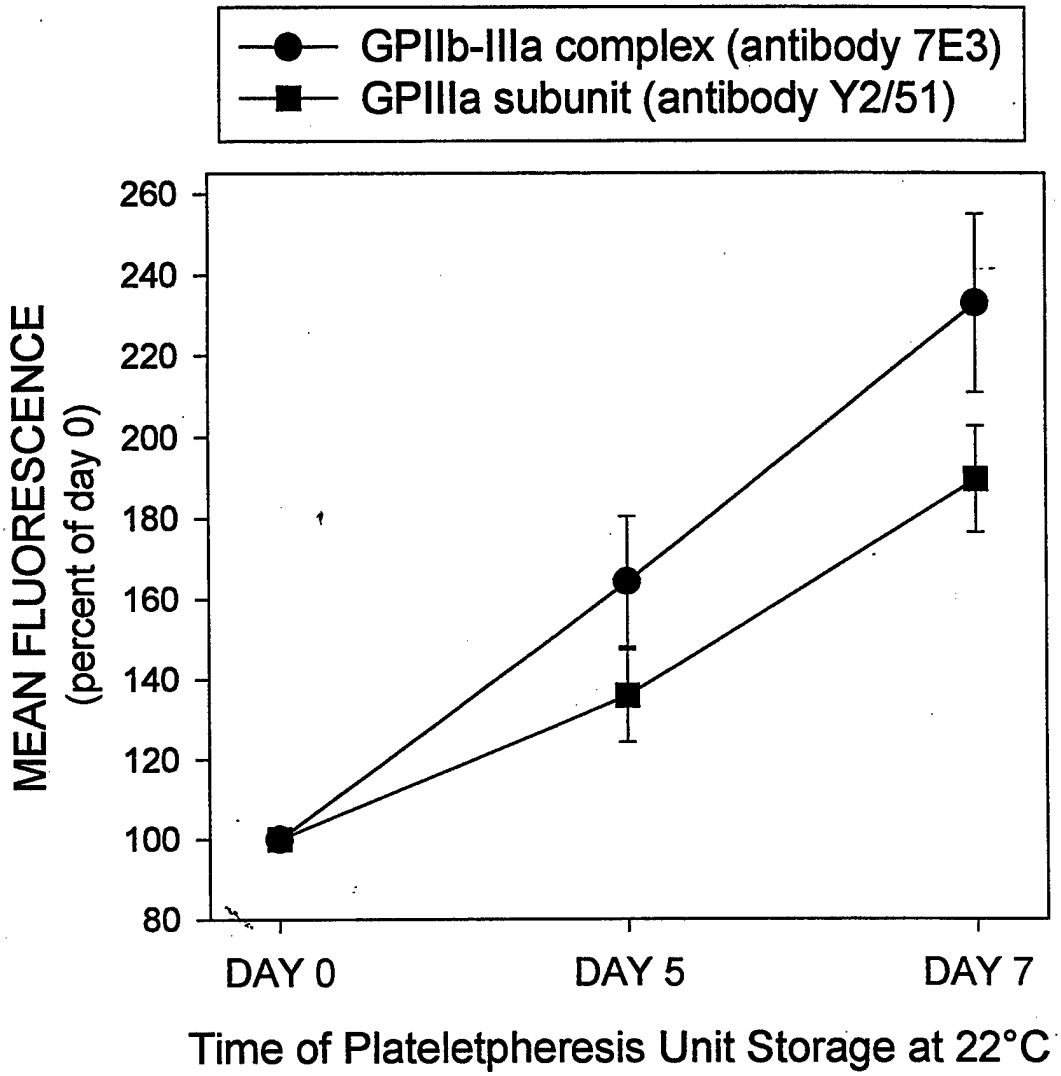

GPIIb-IIIa complex (antibody 7E3)
GPIIIa subunit (antibody Y2/51)
MEAN FLUORESCENCE
(percent of day 0)
260
240
220
200
180
160
140
120
100
80
DAY 0
DAY 5
DAY 7
Time of Plateletpheresis Unit Storage at 22°C

FIGURE 10

Effect of 22°C liquid storage on the activated GPIIb-IIIa complex, as detected by monoclonal antibody PAC1. Plateletpheresis product samples were incubated with a combination of calcium, ADP and epinephrine, or calcium and human α-thrombin (both in the presence of GPRP), or no added agonist (buffer only) for 15 minutes. Platelets and PDMP were identified with anti-CD61 antibody. Light scatter gates were set to include both platelets and PDMP. Maximally activated (ADP-epinephrine-calcium) day 0 plateletpheresis product sample PAC-1 binding was assigned 100 fluorescence units. Data are mean ± SEM, n=6.

FIGURE 10

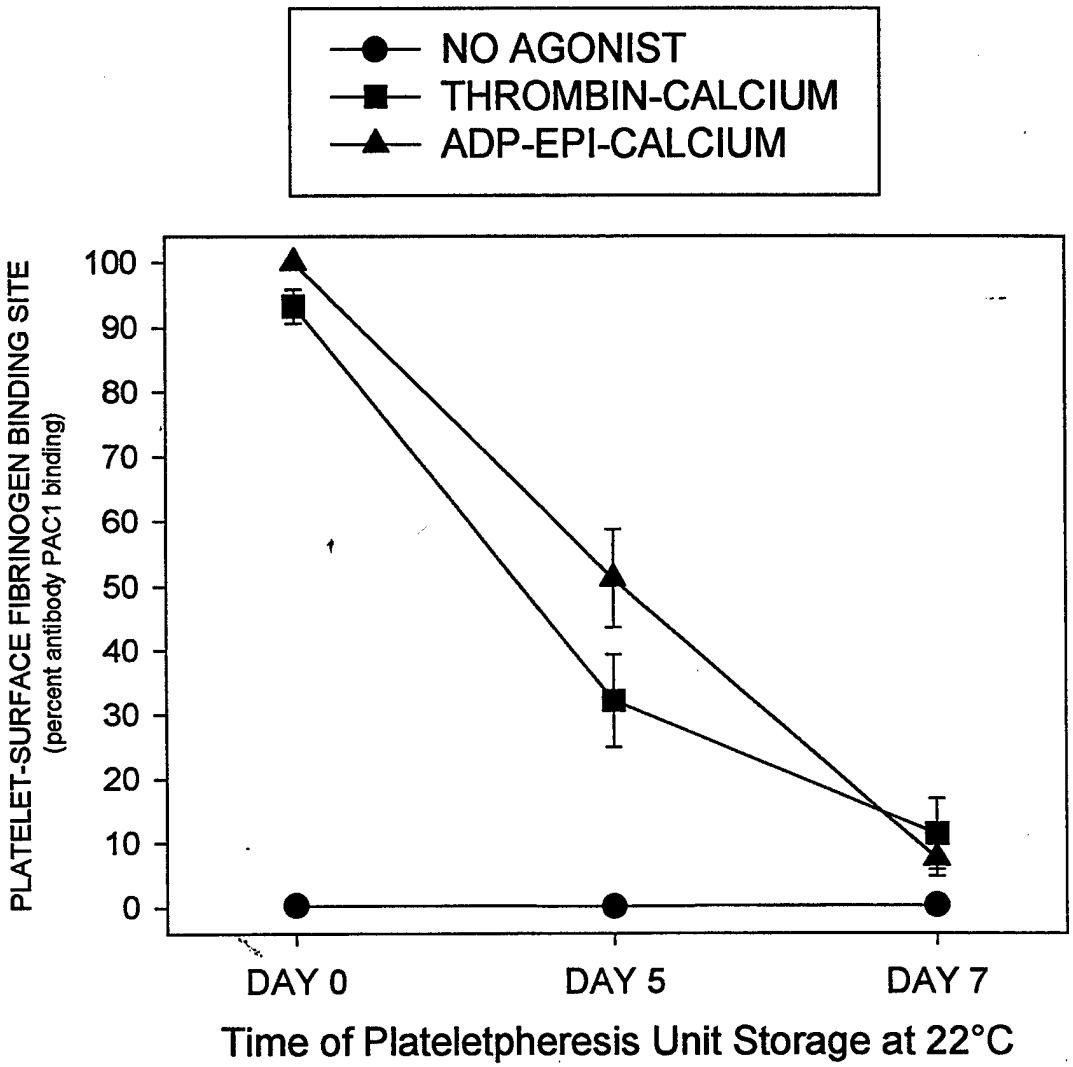

NO AGONIST
THROMBIN-CALCIUM
ADP-EPI-CALCIUM
PLATELET-SURFACE FIBRINOGEN BINDING SITE
(percent antibody PAC1 binding)
100
90
80
70
60
50
40
30
20
10
0
DAY 0
DAY 5
DAY 7
Time of Plateletpheresis Unit Storage at 22°C

FIGURE 11

Representative fluorescence histogram overlays from plateletpheresis products on days 0, 5 and 7 of 22°C liquid storage. Gates were set to include both platelets and PDMP. All samples were activated with ADP-epinephrine-calcium and labeled with antibody PAC1 directed against the fibrinogen binding site of activated GPIIb-IIIa. An emerging agonist-unresponsive subpopulation of platelets can be seen to the left side of each overlay developing on days 5 and 7 of storage. Donor 2 (bottom panel) platelets are nearly all unresponsive by day 7.

FIGURE 11

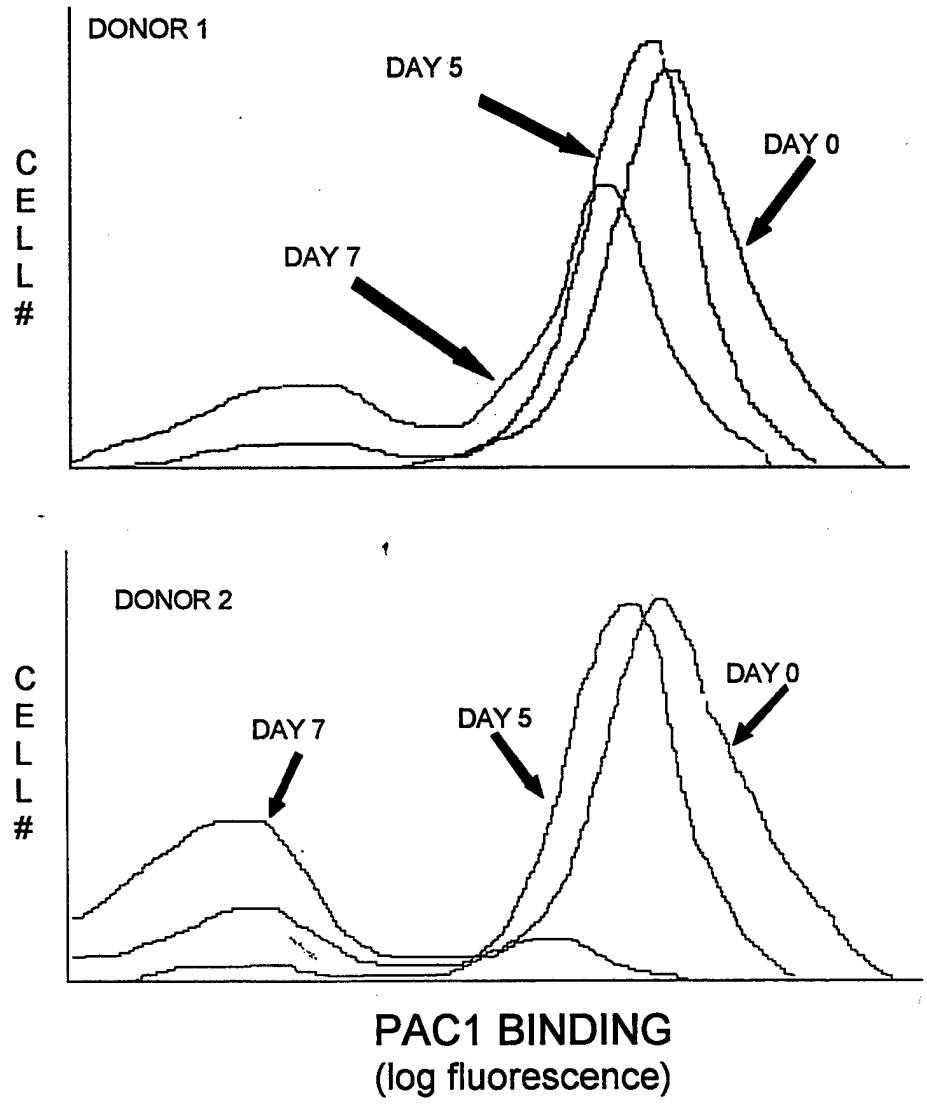

DONOR 1
DAY 5
DAY 0
DAY 7
CELL #
DONOR 2
DAY 0
DAY 7
DAY 5
CELL #
PAC1 BINDING
(log fluorescence)

FIGURE 12

Representative light scatter plots from a 22°C liquid preserved plateletpheresis product and a cryopreserved plateletpheresis product pre-freeze and post-thaw. Platelets and PDMP were identified with anti-CD41/61 antibody.

FIGURE 12

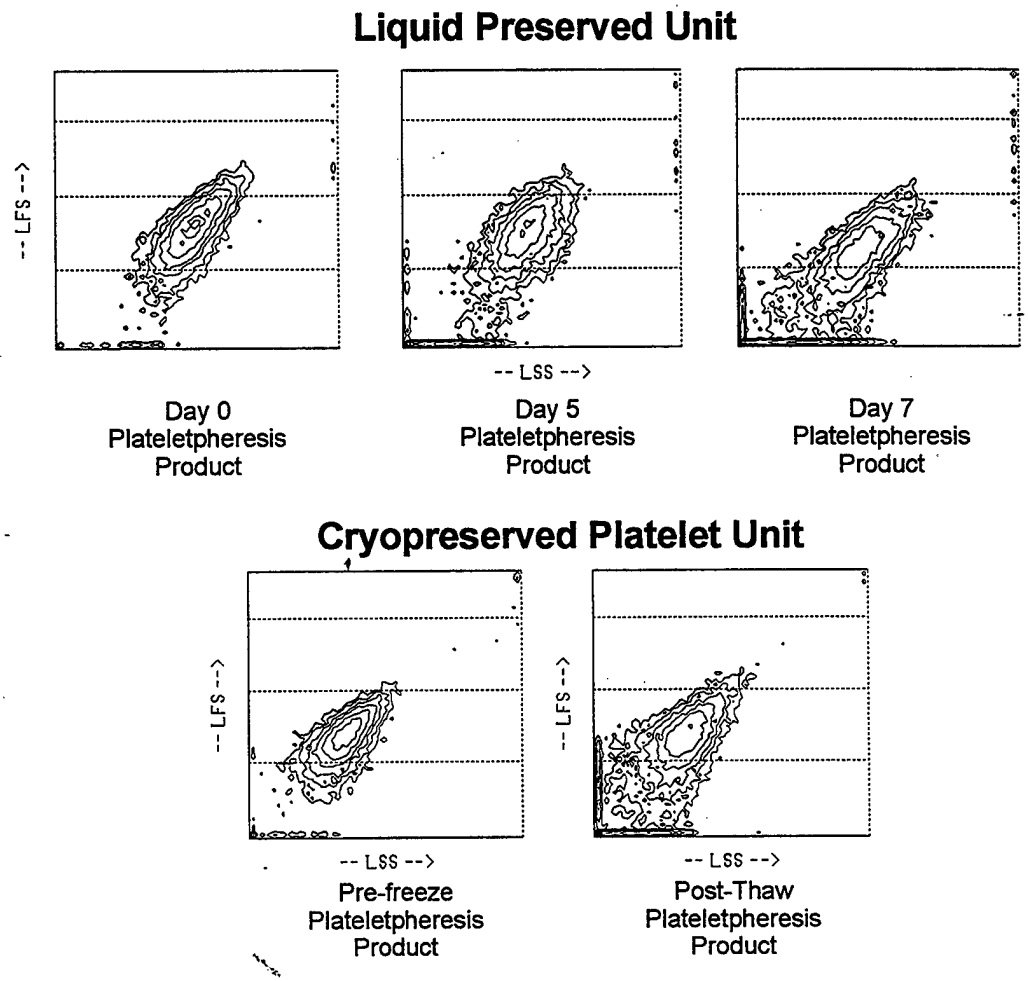

Liquid Preserved Unit
-- LFS -->
-- LSS -->
Day 0 Plateletpheresis Product
Day 5 Plateletpheresis Product
Day 7 Plateletpheresis Product
Cryopreserved Platelet Unit
-- LFS -->
-- LSS -->
Pre-freeze Plateletpheresis Product
-- LFS -->
-- LSS -->
Post-Thaw Plateletpheresis Product

FIGURE 13

Representative light scatter plots from a cryopreserved plateletpheresis product. Region A constitutes platelet aggregates and region M constitutes platelet microparticles. A portion of the microparticles produced by the freeze-thaw process are removed by the wash procedure (compare the lower 2 panels). Platelets and PDMP were identified with anti-CD41/61 antibody.

FIGURE 13

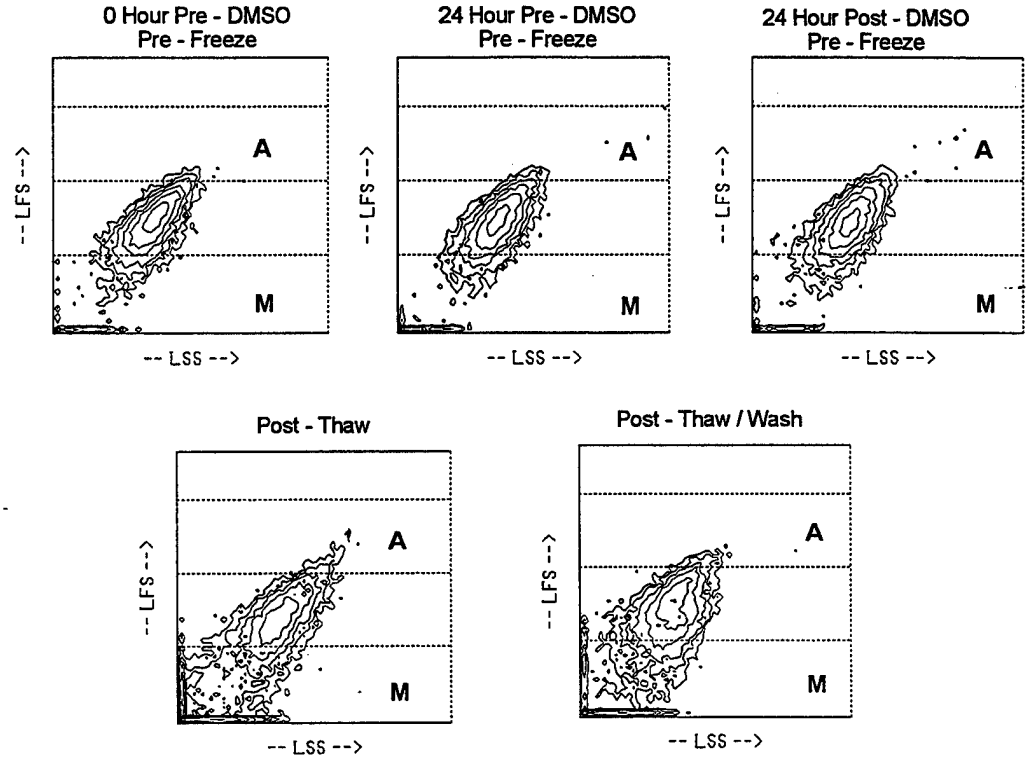

0 Hour Pre - DMSO
Pre - Freeze
24 Hour Pre - DMSO
Pre - Freeze
24 Hour Post - DMSO
Pre - Freeze
Post - Thaw
Post - Thaw / Wash
A
M
-- LFS -->
-- LSS -->

FIGURE 14

Effect of cryopreservation on platelet aggregate and microparticle formation. Unit samples were compared to the donors' peripheral blood platelets to establish log forward light scatter (LFS) regions corresponding to platelet-specific events of increased (aggregated) LFS. Platelet derived microparticles (PDMP) were quantitated by the factor V binding method (figure 2). All samples were prepared with no added agonist. Platelets and PDMP were identified by binding of anti-CD41/61 antibody. Data are mean ± SEM, n=7.

FIGURE 14

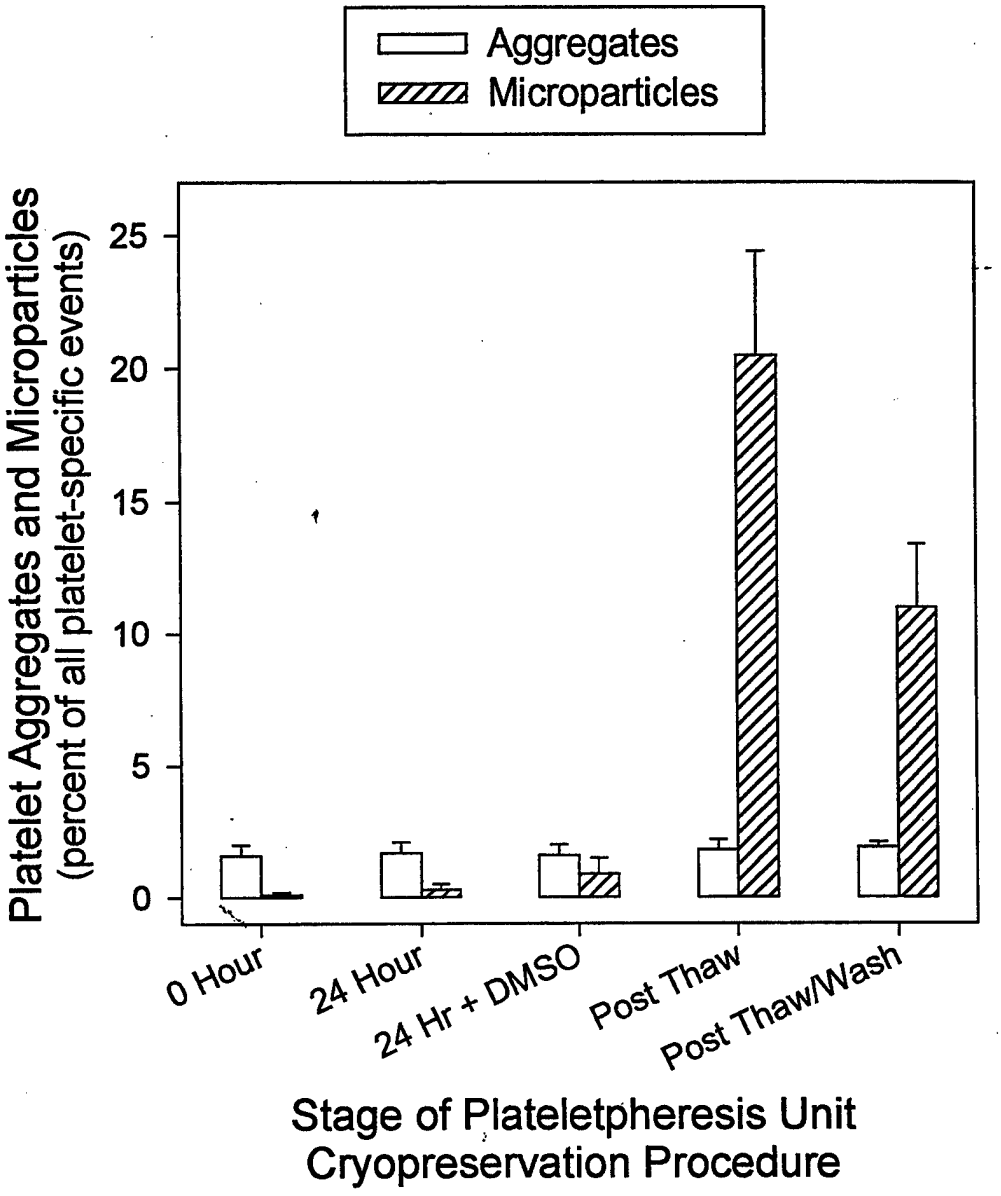

Aggregates
Microparticles
Platelet Aggregates and Microparticles
(percent of all platelet-specific events)
25
20
15
10
5
0
0 Hour
24 Hour
24 Hr + DMSO
Post Thaw
Post Thaw/Wash
Stage of Plateletpheresis Unit
Cryopreservation Procedure

FIGURE 15

Representative light scatter plots from a normal plateletpheresis product (panel A) and a plateletpheresis product containing microaggregates (panel B). Note the characteristic "tail" produced by the microaggregates in the upper right portion of histogram B. Platelets, platelet aggregates, and PDMP were identified with anti-CD41/61 antibody.

FIGURE 15

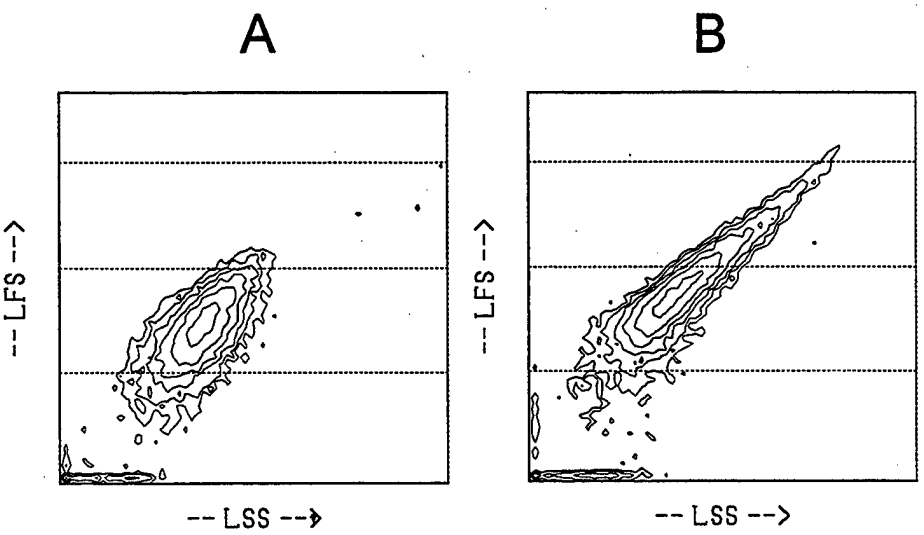


FIGURE 16

Effect of cryopreservation on platelet surface P-selectin. An analysis region was made to include 1% positive events using an irrelevant isotype-matched mouse IgG. The same region was used on subsequent samples labeled with the P-selectin-specific monoclonal antibody S12. Data are expressed as the percentage of all platelet-specific events in this region using a sample with no added agonist. Platelets and PDMP were identified by binding of anti-CD41/61 antibody. Light scatter gates were set to include both platelets and PDMP. Data are mean ± SEM, n=7.

FIGURE 16

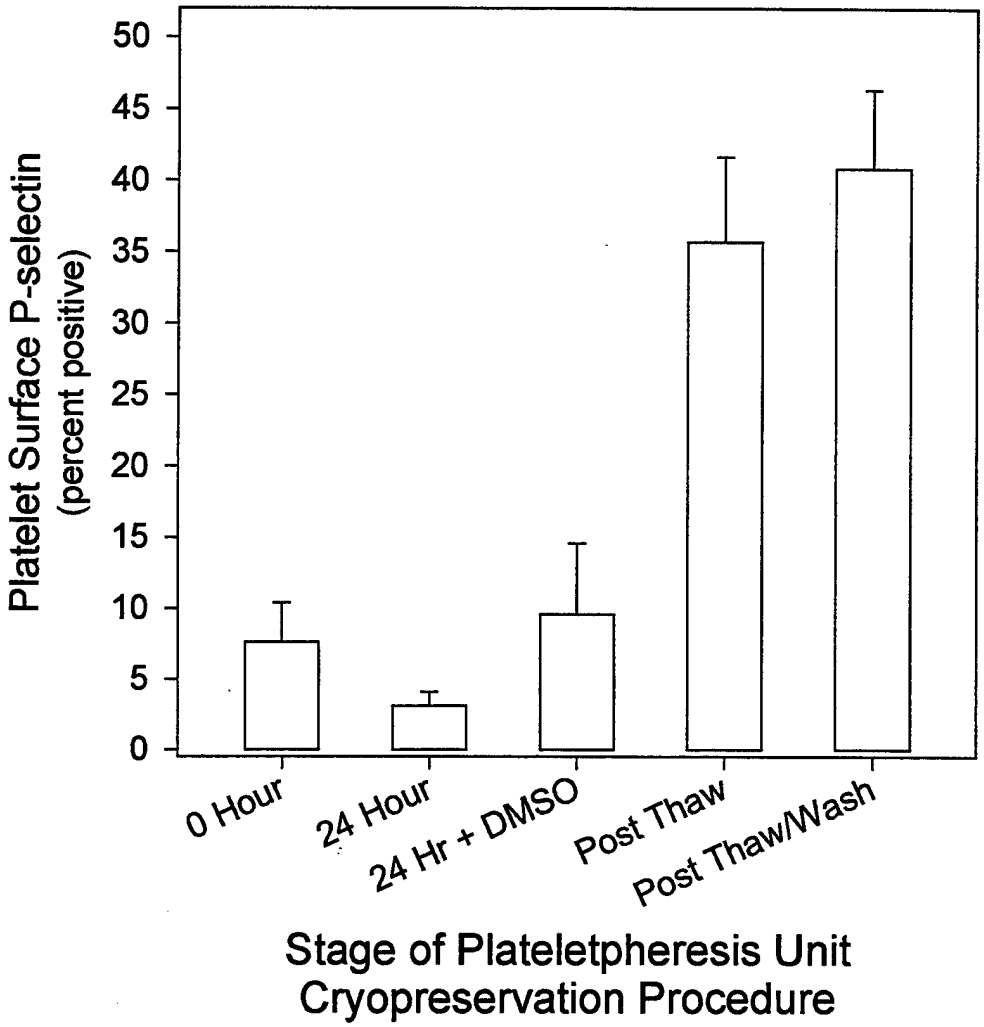

Platelet Surface P-selectin
(percent positive)
50
45
40
35
30
25
20
15
10
5
0
0 Hour
24 Hour
24 Hr + DMSO
Post Thaw
Post Thaw/Wash
Stage of Plateletpheresis Unit
Cryopreservation Procedure

FIGURE 17

Effect of cryopreservation on platelet reactivity to thrombin as reported by surface P-selectin. Time 0 and post thaw-wash cryopreserved plateletpheresis product samples were incubated with a) 2 U/mL human α-thrombin in the presence of GPRP or b) buffer only. Separate aliquots were labeled with anti-P-selectin antibody (S12) or an irrelevant control IgG. An analysis region was made to include 1% positive events using the irrelevant isotype-matched mouse IgG. The same region was used on subsequent samples labeled with antibody S12 against P-selectin. Data are expressed as the percentage of all platelet-specific events in this region after subtracting the control IgG value. Platelets and PDMP were identified by binding of anti-CD41/61 antibody. Light scatter gates were set to include both platelets and PDMP. Data are mean ± SEM, n=7.

FIGURE 17

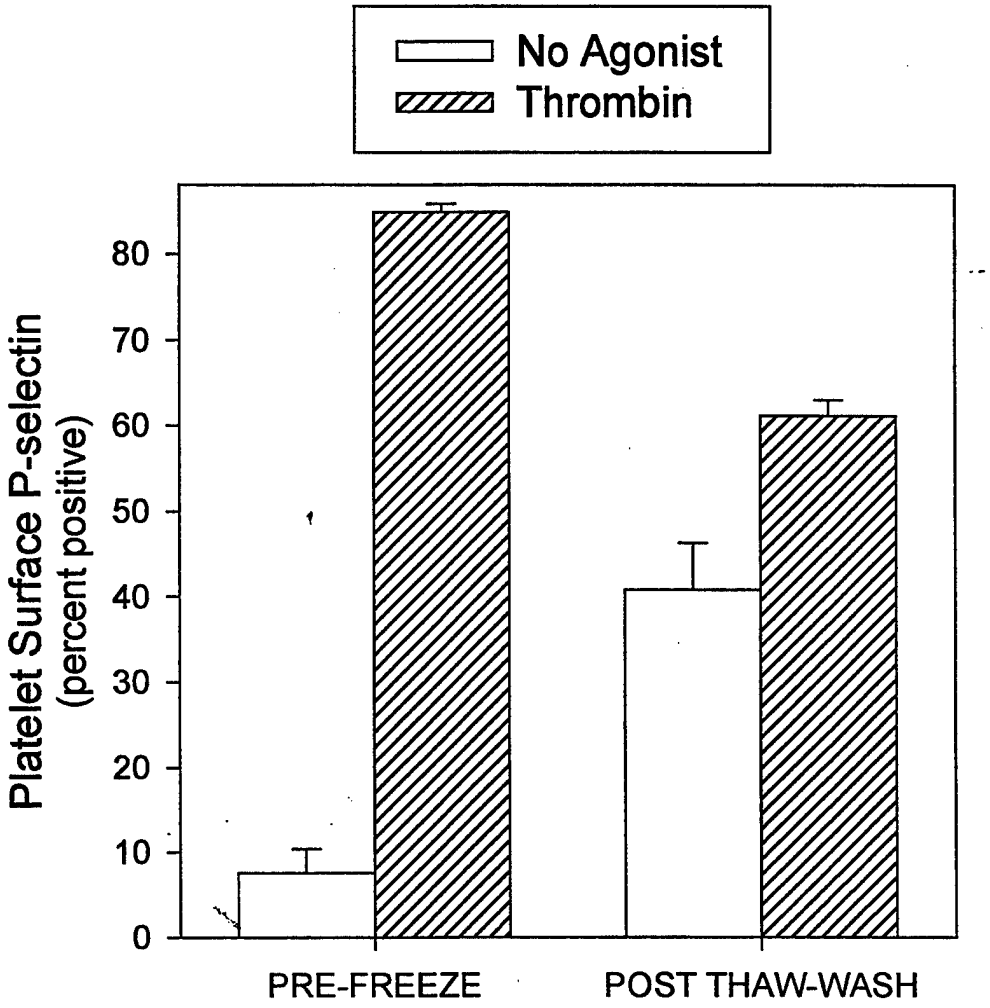

No Agonist
Thrombin
Platelet Surface P-selectin
(percent positive)
0
10
20
30
40
50
60
70
80
PRE-FREEZE
POST THAW-WASH

FIGURE 18

Effect of cryopreservation on platelet surface GPIb. Samples were labeled with the GPIb-specific antibody 6D1. Data are expressed as the number of antibody molecules bound, using QSC beads to generate a standard curve of fluorescence vs. antibody binding. All plateletpheresis product samples were prepared with no added agonist. Platelets and PDMP were identified by binding of anti-CD41/61 antibody. Light scatter gates were set to include both platelets and PDMP. Data are mean ± SEM, n=7.

FIGURE 18

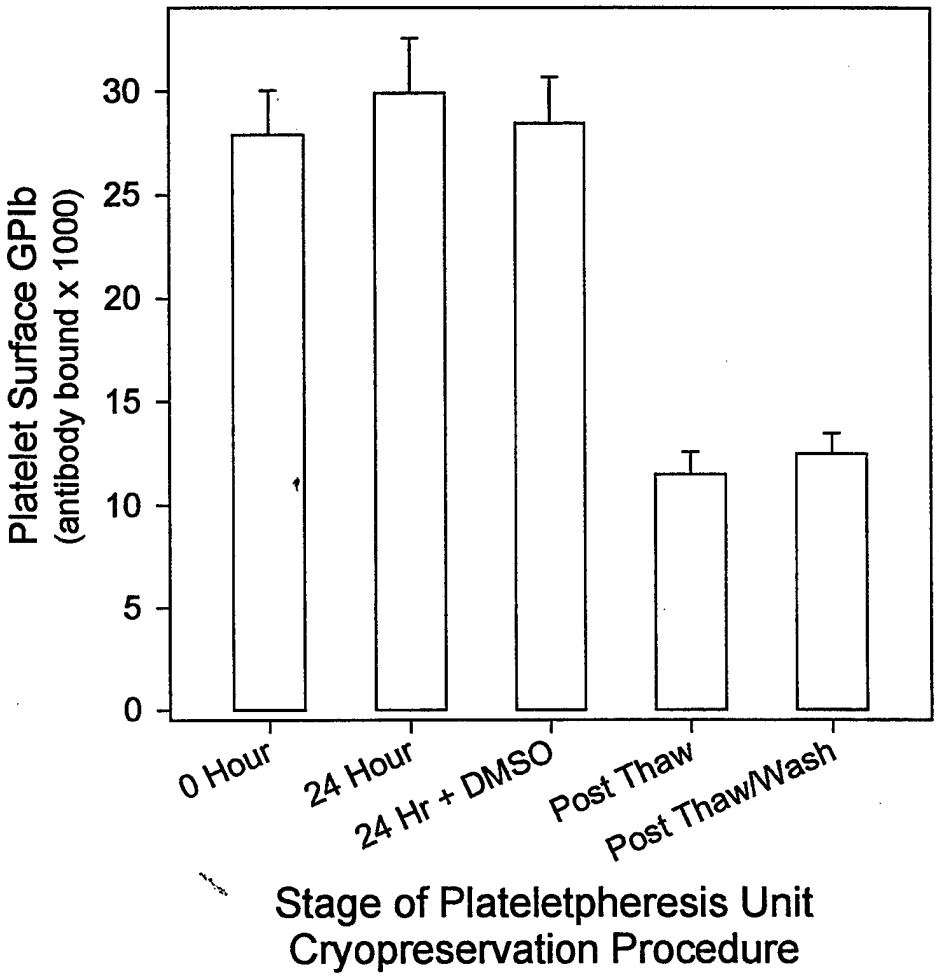

Platelet Surface GPIb
(antibody bound x 1000)
30
25
20
15
10
5
0
0 Hour
24 Hour
24 Hr + DMSO
Post Thaw
Post Thaw/Wash
Stage of Plateletpheresis Unit
Cryopreservation Procedure

FIGURE 19

Effect of cryopreservation on platelet reactivity to thrombin, as reported by the decrease in surface GPIb. Time 0 and post thaw-wash cryopreserved plateletpheresis product samples were incubated with a) 2 U/mL human α-thrombin in the presence of GPRP or b) buffer only. Samples were labeled with the GPIb-specific monoclonal antibody 6D1. Data are expressed as the number of antibody molecules bound, using QSC beads to generate a standard curve of fluorescence vs. antibody binding. Platelets and PDMP were identified by binding of anti-CD41/61 antibody. Light scatter gates were set to include both platelets and PDMP. Data are mean ± SEM, n=7.

FIGURE 19

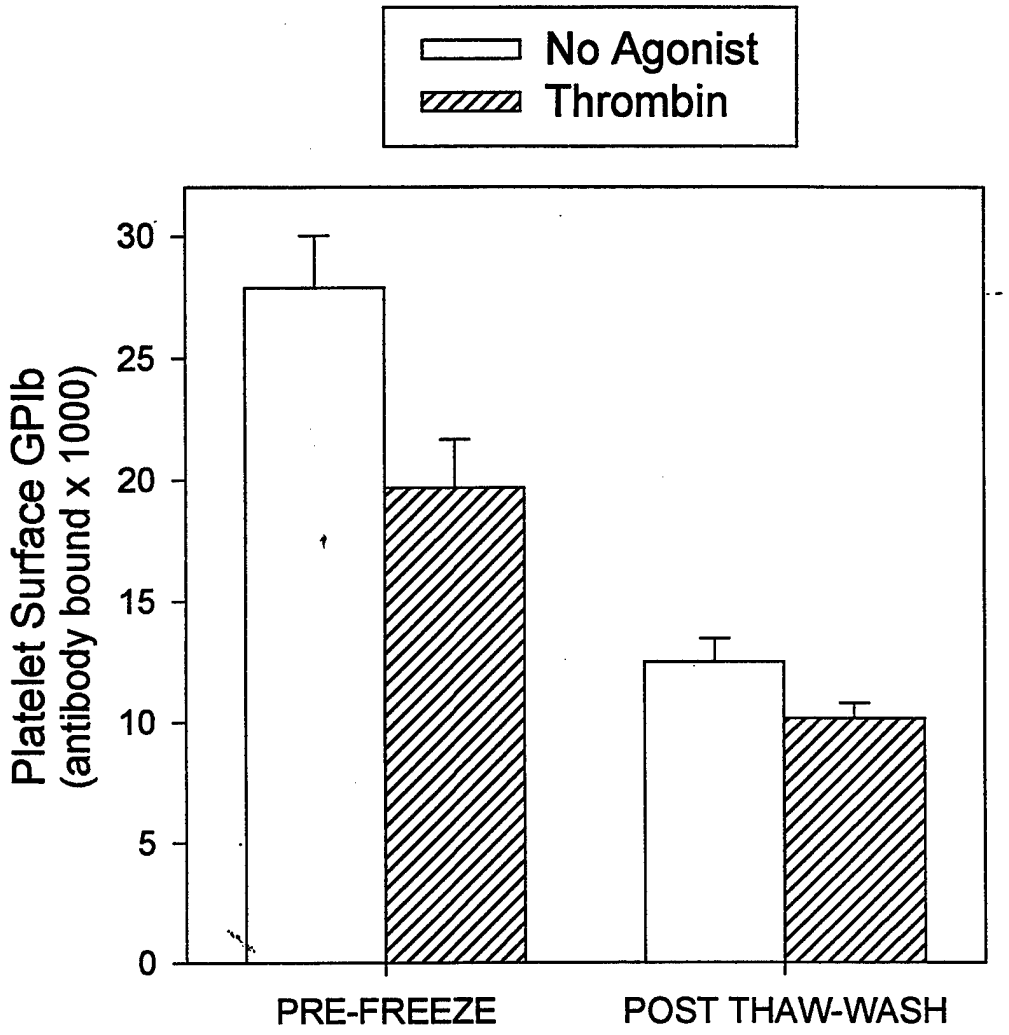

No Agonist
Thrombin
Platelet Surface GPIb
(antibody bound x 1000)
30
25
20
15
10
5
0
PRE-FREEZE
POST THAW-WASH

FIGURE 20

Representative two-dimensional histogram from a post-thaw/wash cryopreserved plateletpheresis product. The subpopulation which has low surface GPIb (region A) also has relatively higher surface bound factor V. The subpopulation which has normal surface GPIb (region B) has generally lower surface bound factor V, but a portion of these platelets are expressing increased surface bound factor V (top of region B). This plateletpheresis product sample was resuspended in autologous plasma without added agonist. Platelets and PDMP were identified with anti-CD61 antibody.

FIGURE 20

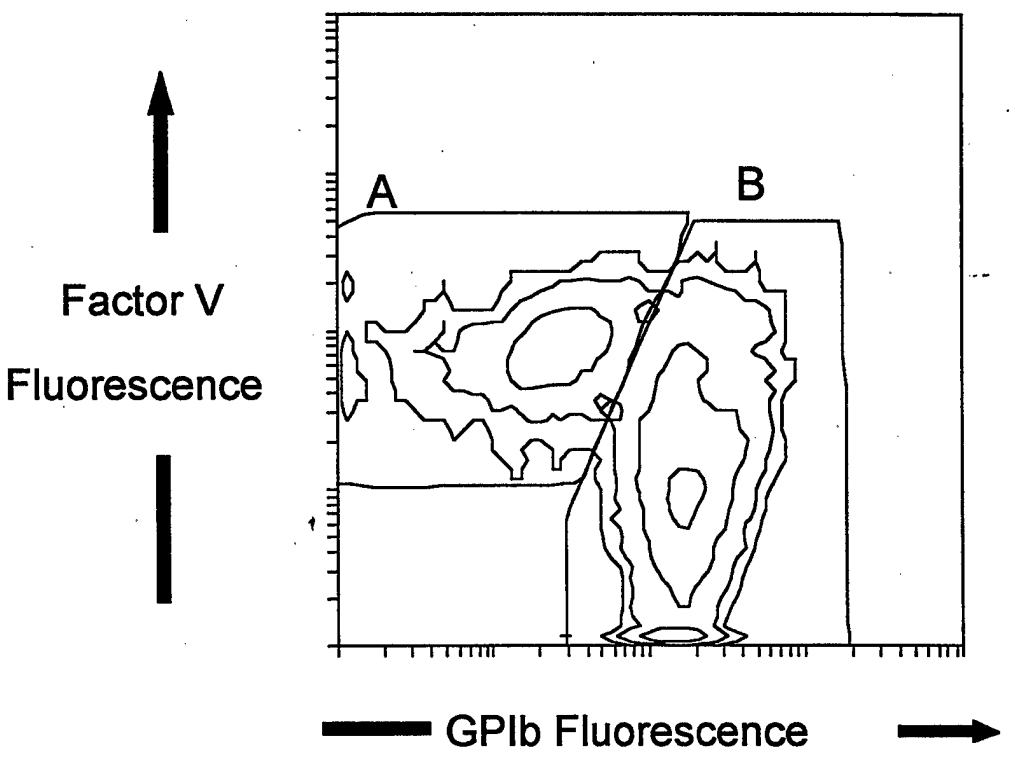


FIGURE 21

The GPIb reduced subpopulation of post-thaw/wash cryopreserved platelets has more surface-bound coagulation factor V than the GPIb normal subpopulation. Platelets and PDMP were identified by binding of anti-CD61 antibody. Light scatter gates were set to include both platelets and PDMP. Data are mean ± SEM, n=3.

FIGURE 21

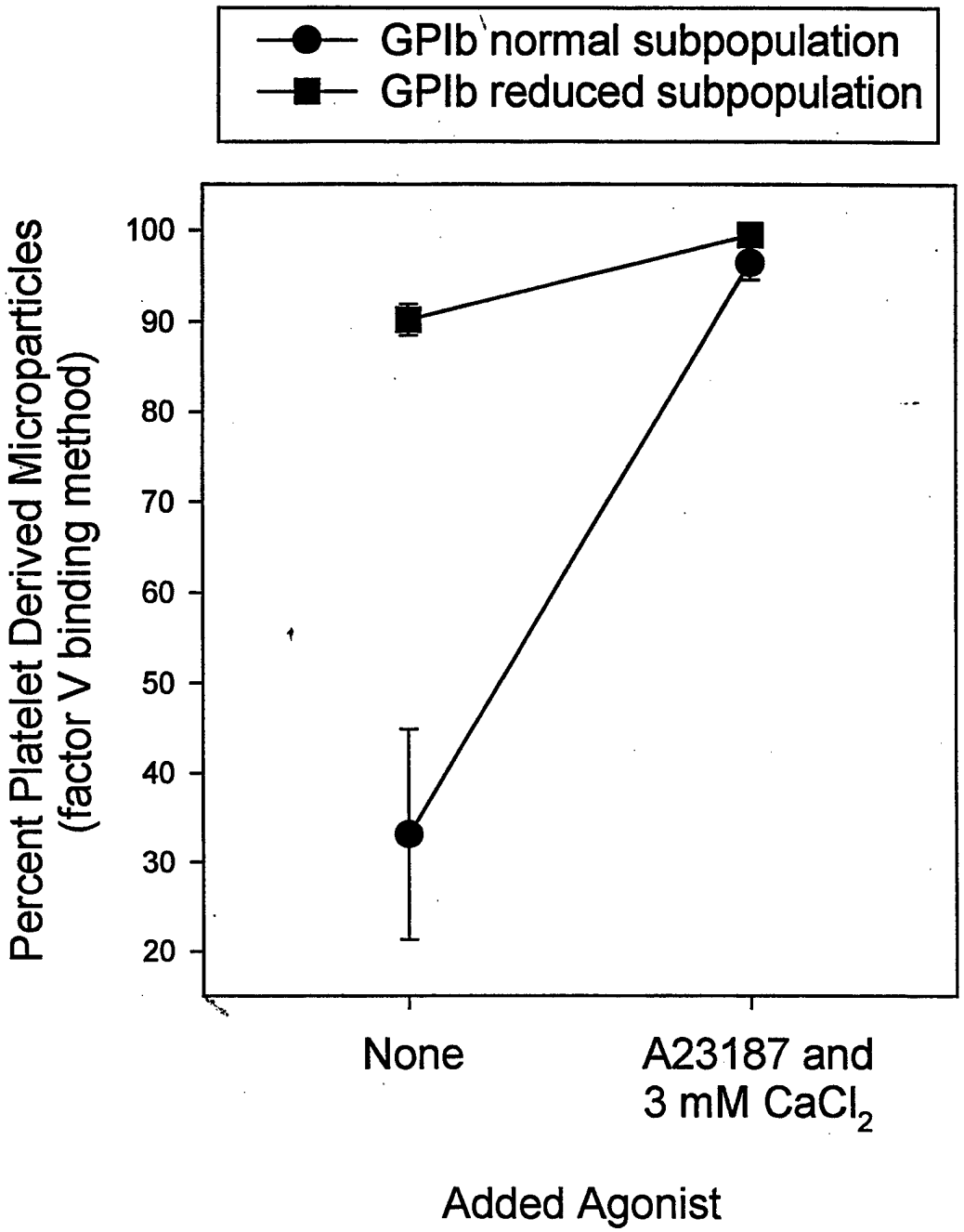

GPIb normal subpopulation
GPIb reduced subpopulation
Percent Platelet Derived Microparticles
(factor V binding method)
100
90
80
70
60
50
40
30
20
None
A23187 and
3 mM CaCl2
Added Agonist

FIGURE 22

The GPIb normal subpopulation of post-thaw/wash cryopreserved platelets is more responsive to thrombin activation than the GPIb reduced subpopulation as reported by the P-selectin-specific monoclonal antibody S12. Platelets and PDMP were identified by binding of anti-CD61 antibody. Light scatter gates were set to include both platelets and PDMP. Data are mean ± SEM, n=7.

FIGURE 22

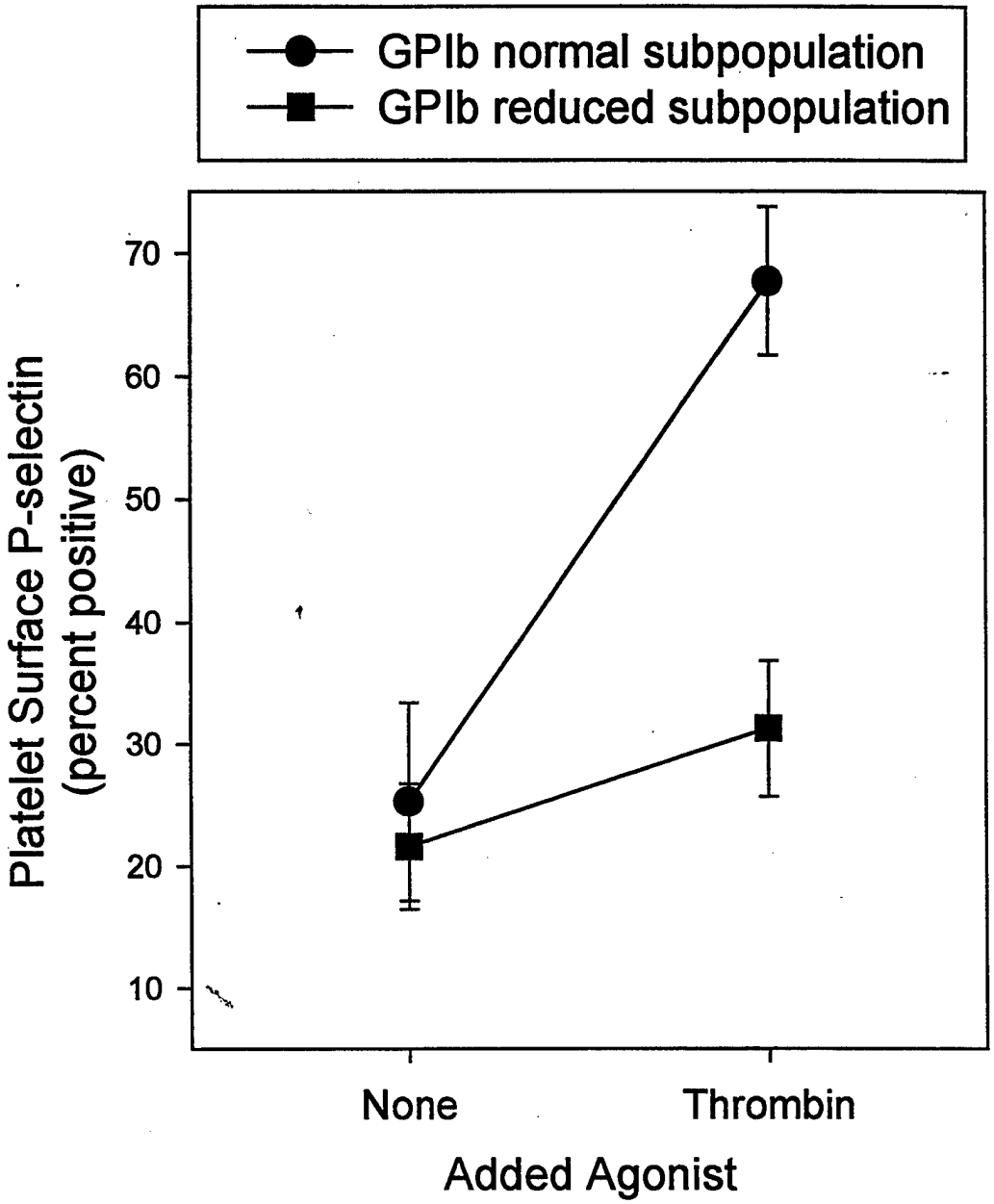
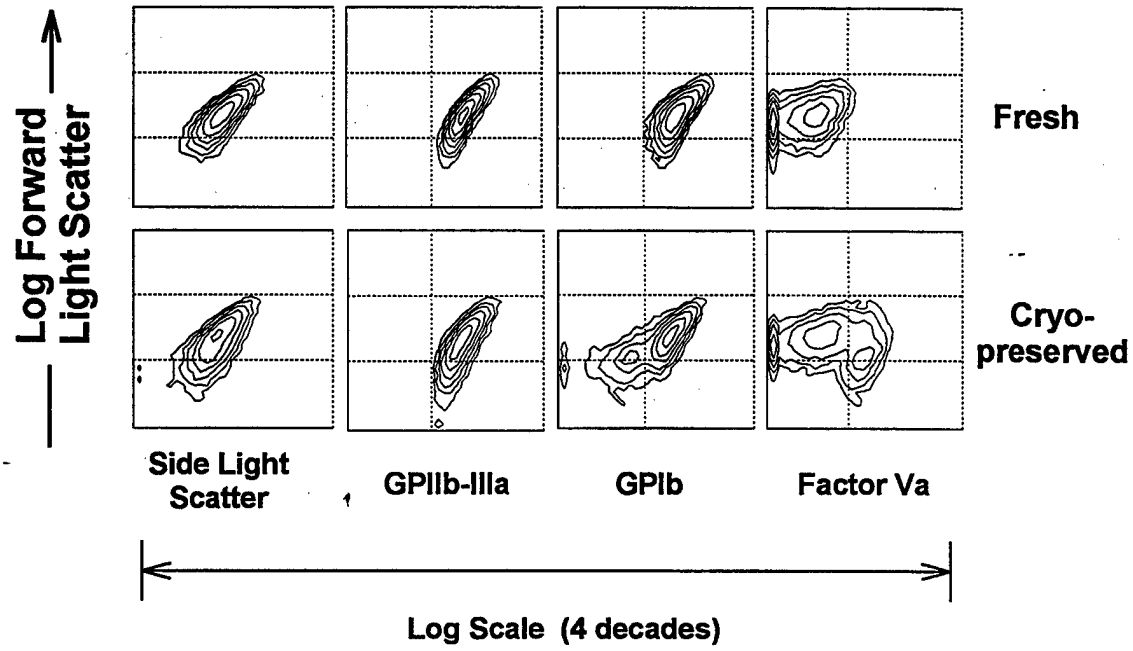

GPIb normal subpopulation
GPIb reduced subpopulation
Platelet Surface P-selectin
(percent positive)
70
60
50
40
30
20
10
None
Thrombin
Added Agonist

FIGURE 23

Multiparameter flow cytometry of fresh platelets and post-thaw/wash cryopreserved platelets. Using 2 light scatter parameters and 3 color fluorescence analysis, flow cytometry was used to generate each of these 2 sets of histograms simultaneously. These contour histograms were smoothed to allow better visual discrimination of individual subpopulations. Light scatter gates were set to include both platelets and PDMP. Constitutive platelet surface GPIIb-IIIa was measured with monoclonal antibody 7E3. Platelet surface GPIb was measured using monoclonal antibody 6D1. Platelet surface-bound factor V was measured by monoclonal antibody V237. The cryopreserved platelets include a subpopulation which has reduced surface GPIb and increased surface bound coagulation factor V. These measurements coupled with log forward light scatter (Y-axis) indicate the GPIb reduced and increased factor V subpopulations have a decreased forward light scatter signal.

FIGURE 23


Log Forward Light Scatter
Fresh
Cryo-preserved
Side Light Scatter
GPIIb-IIIa
GPIb
Factor Va
Log Scale (4 decades)